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Publication date:
2013

Document Version
Early version, also known as pre-print

Citation for published version (APA):
Dai, L. (2013). *Toxicity and bioaccumulation of silver and copper oxide nanoparticles in two deposit feeders, a polychaete Capitella teleta and a mullusk, Macoma balthica, compared to other metallic forms*. Roskilde Universitet.

Toxicity and bioaccumulation of silver and copper oxide nanoparticles in two deposit feeders, a polychaete *Capitella teleta* and a mollusk, *Macoma balthica*, compared to other metallic forms

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PhD Thesis

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December, 2012

Cover story:
Layout: Ritta Juel Bitsch and Lina Dai
Front page: Ritta Juel Bitsch and Lina Dai
Print: Printfo Paritas Digital Service

ISBN 978-87-7349-832-3

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Abstract

Expanding applications of engineered nanoparticles in industrial and consumer products has led to rapid development of nanotechnology and thus in products containing nanoparticles. It has been reported that engineered metal-bearing nanoparticles can reach the aquatic environment and end up in sediment mainly from sewage treatment plants. Therefore, there is an increasing need for understanding ecotoxicity and environmental risks of engineered nanoparticles to deposit feeders. Both silver and copper oxide nanoparticles were chosen because of their wide applications resulting from their effective antibacterial properties.

In the present thesis, both silver and copper oxide nanoparticles (Ag NPs and CuO NPs) were studied in two marine deposit feeders, a polychaete, *Capitella teleta,* and a mollusk, *Macoma balthica*. Most of the nanoparticles used were synthesized by partners from the EU FP7 NanoReTox project with strict control on nanoparticle properties, such as shape and size. The rest of the nanoparticles used (mainly for the clam exposure) were from a commercial source and were characterized by the National History Museum before exposures.

Nanoparticles toxicity were assessed by measuring mortality, growth, feeding rate, conditional index, and DNA damage. Bioaccumulation of the nanoparticles was determined by an uptake and depuration kinetic method in which net uptake rates and depuration rates were quantified based on changes in metal nanoparticle body burden over time.

In order to determine whether bioaccumulation or toxic effects were driven by particle form (incl. both nanoparticles and micron-sized particles), particle size or other characteristics (i.e., released ions), exposure to the same metal in both ionic form and bulk form (refers to particles with size larger than 100 nm in the present thesis) were compared. In addition, diffusive gradient in thin films (DGT) were tested on a small scale to study nanoparticle dissolution in sediment.

The overall results demonstrated that: 1) no toxic effects of nanoparticles were detected on the selected endpoints, except for a delayed mortality of *C. teleta* after 7 d of exposure to all Cu/CuO treatments; 2) nanoparticles in sediment were bioavailable and were bioaccumulated in both species; 3) species-specific

bioaccumulation patterns were observed such that bioaccumulation in *C. teleta* depended on particle shape but not size or form whereas bioaccumulation in *M. balthica* showed a strong decreasing trend with increasing size (i.e., ions > small nanoparticles > big nanoparticles > bulk). Whereas body burden in *C. teleta* reduced to background levels after 7 d of depuration, there was minimal depuration of Cu in particle form in *M. balthica*. These results suggest that different mechanisms may be involved in bioaccumulation of metal nanoparticles in these species.

Keywords: Silver nanoparticles, Copper oxide nanoparticles, Metallic form, Metal ions, Deposit feeders, Polychaete, *Capitella teleta*, *Macoma balthica*, Invertebrates, Marine sediment, Toxicity, Bioavailability, Bioaccumulation, Uptake and depuration kinetics

Abstract-Danish

Den stigende anvendelse af kunstigt fremstillede nanopartikler (NPer) i industri- og forbrugerprodukter har ført til en hurtig udvikling af nanoteknologi og dermed produkter, der indeholder NPer. Det er blevet rapporteret, at metalholdige NPer bliver udledt til vandmiljøet hovedsageligt fra rensningsanlæg for at ende i sedimentet. Derfor er der et stigende behov for at forstå økotoksiciteten og de miljømæssige risici der er forbundet med kunstigt fremstillede NPer til sedimentædende organismer. Både sølv og kobberoxid NPer blev valgt på grund af deres brede anvendelse som følge af deres effektive antibakterielle egenskaber.

I den foreliggende afhandling, blev både sølv (Ag) og kobberoxid (CuO) NPer tilsat til sediment undersøgt i to marine sedimentædende organismer, en polychaete, *Capitella teleta*, og en mollusk, *Macoma balthica*. De fleste af de anvendte NPer blev syntetiseret af partnere fra EU FP7 NanoReTox projektet under streng kontrol med NPernes egenskaber, såsom form og størrelse. De resterende NPer, der blev anvendt (hovedsagelig til muslinge eksponering) var fra en kommerciel kilde og blev karakteriseret ved Natural History Museum, UK, før anvendelse.

NP toksicitet blev undersøgt ved at måle dødelighed, vækst, æderate, konditionsindeks, og DNA-skader. Biologisk akkumulering af metal NPer blev bestemt ved at undersøge optagelse og udskillelses kinetik. Her blev netto optagelses- og udskillelsesrater kvantificeret baseret på ændringer i metal NP kropsbyrde over tid. For at undersøge om bioakkumulering og/eller toksiske virkninger blev drevet af partikel form (inkl. både NPer og mikropartikler), partikelstørrelse eller andre egenskaber (dvs. frigivelse af ioner) blev det samme metal tilsat både som ionform og som 'bulk' form (refererer til partikler med størrelse større end 100 nm i denne afhandling). Endvidere blev der anvendt Diffusive Gradient Thin (DGT) membraner til at teste frigivelsen af ioner fra NPer i sedimentet.

De overordnede resultater viste at: 1) der ikke var signifikant effekt af metal NP mikset i sedimentet på de udvalgte endpoints, men at der blev observeret forsinket dødelighed af *C. teleta* efter 7 dages eksponering til alle Cu/CuO behandlinger, 2) metal tilført sediment som NPer var biotilgængeligt og bioakkumulerede hos både *C. teleta* og *M. baltica*, og 3) der var artsspecifikke variationer i bioakkumulering, således at bioakkumulering i *C. teleta* afhang af partikelform, men ikke af størrelse,

hvorimod bioakkumulering i *M. balthica* viste en stærk faldende tendens med stigende størrelse (dvs. Ioner > små NPer > store NPer > bulk). I *C. teleta* blev kropsbyrde reduceret til baggrundsniveauer efter 7 dage i rent sediment, mens der hos *M. balthica* kun blev observeret minimal udskillelse af Cu i forsøg med partikulær kobber. Disse resultater tyder på, at forskellige mekanismer kan være involveret i bioakkumulering af metal NPer i disse arter.

Nøgleord: Sølv nanopartikler, kobberoxid nanopartikler, Metal form, metalioner, sedimentæder, Polychaete, *Capitella teleta*, *Macoma balthica*, Invertebrater, Marint sediment, Toksicitet, biotilgængelighed, Bioakkumulering, kinetik

1. Introduction

Since the nanotechnology industry began its development less than a few decades ago, engineered metal nanoparticles (ENPs) and nanomaterials have been applied widely in our daily lives in a range of products and applications including scratchproof eyeglasses, anti-graffiti coatings for walls and transparent sunscreens, food safety and packaging, to medicine (http://ec.europa.eu/health/opinions2/en/nanotechnologies). The number of consumer products containing ENPs on the market is expected to reach 3400 by 2020 under current trends (http://www.nanotechproject.org/news/archive/9231/). The market for nanotechnologies was estimated at $700 billion by 2008 and will reach more than $1 trillion by 2015 (US National Science Foundation, 2007). Given the wide use of ENPs, nanomaterials and nanotechnologies, and the increasing importance of this field in the economy, there is a need to regulate nanotechnologies and to assure safe use and disposal of ENPs and nanomaterials.

Concerns about the safety of ENPs and nanomaterials for both human health and the environment have been raised. ENPs refer to particles produced with at least one dimension between 1 – 100 nm (The Royal Society & The Royal Academy of Engineering 2004). They can be designed and produced with different particle sizes, shapes and compositions in order to meet the need of applications to different areas. Due to these geometric and chemical changes, novel physical chemical properties of ENPs arise, such as high reactivity due to an increasing ratio of surface area to volume. This may cause very different effects and toxicities for nanoparticles compared to bulk particles (particles larger than 100 nm) of the same composition. However, it is unclear whether current risk assessment methodologies are sufficient for assessing ENPs. Some uncertainties caused by particle form, nanoparticle size and nanoparticle shape in the identification of intrinsic toxicities, internal and external exposure levels, and toxicokinetics have been summarized by EUROPA (http://ec.europa.eu/health/opinion s2/en/nano-

technologies). Hence, the filling of knowledge gaps about ENPs could facilitate the extrapolation from the risk assessment from known benchmarks (e.g., ionic forms of metals) or determine whether there is a need for nano-specific risk assessment. The focus of this thesis is on ENPs; however other ENPs are included as illustrations when relevant and no distinctions are made between nanoparticles and nanomaterials in this thesis.

1.1 Sources, fate and speciation of engineered silver and copper oxide ENPs in the aquatic environment

ENPs enter the aquatic environment mainly via effluents from sewage treatment plants, compared to pathways of spillage, discharge, atmospheric deposition or soil run off (Boxall et al., 2007, Mueller and Nowack, 2008, Blaser et al., 2008). The predicted environmental concentration of silver nanoparticles (Ag NPs) increases from 195 and 1203 ng/kg per year in sediment compartment while no estimations are available yet for copper oxide nanoparticles (CuO NPs) (Gottschalk et al., 2009). In the aquatic environment, the fate of ENPs can be divided into the abiotic processes and biotic processes described in detail below. Primarily, abiotic processes include natural organic matter (NOM) stabilization and flocculation, degradation, dissolution, homo- and hetero- aggregation, photo-degradation and sedimentation (Fig. 1) (Batley et al., 2012, Lowry et al., 2012b). Multi-walled carbon nanotube (50 to 500 mg/L) can disperse evenly in the suwannee river natural organic matter solution (10 to 100 mg/L) over 1 month (Hyung et al., 2007). Sodium chloride concentrations and divalent cations cause the rapid instability of single dispersed silver nanoparticles (Ag NPs) by decreasing negative charges on particle surface (Chinnapongse et al., 2011). Ghosh et al. (2008) detected that the stabilization of aluminum oxide nanoparticles was dependent on both NOM and pH in the presence of sodium chloride. ENPs can also sorb on surfaces of minerals and larger, rigid biopolymers which are classified as natural colloids (Quik et al., 2010). All these processes facilitate the removal of ENPs out of the water phase and their deposition on the surface of sediment where all of the mentioned abiotic processes may occur similarly. On the other hand, aquatic organisms can accumulate ENPs through dietary uptake and make the accumulated ENPs bioavailable to organisms at higher trophic levels (Holbrook et al., 2008, Zhu et al., 2010). For example, exposure to zinc nanoparticles and cerium oxide nanoparticles showed that a suspension-feeding mussel, *Mytilus galloprovincialis*, can biotransform zinc

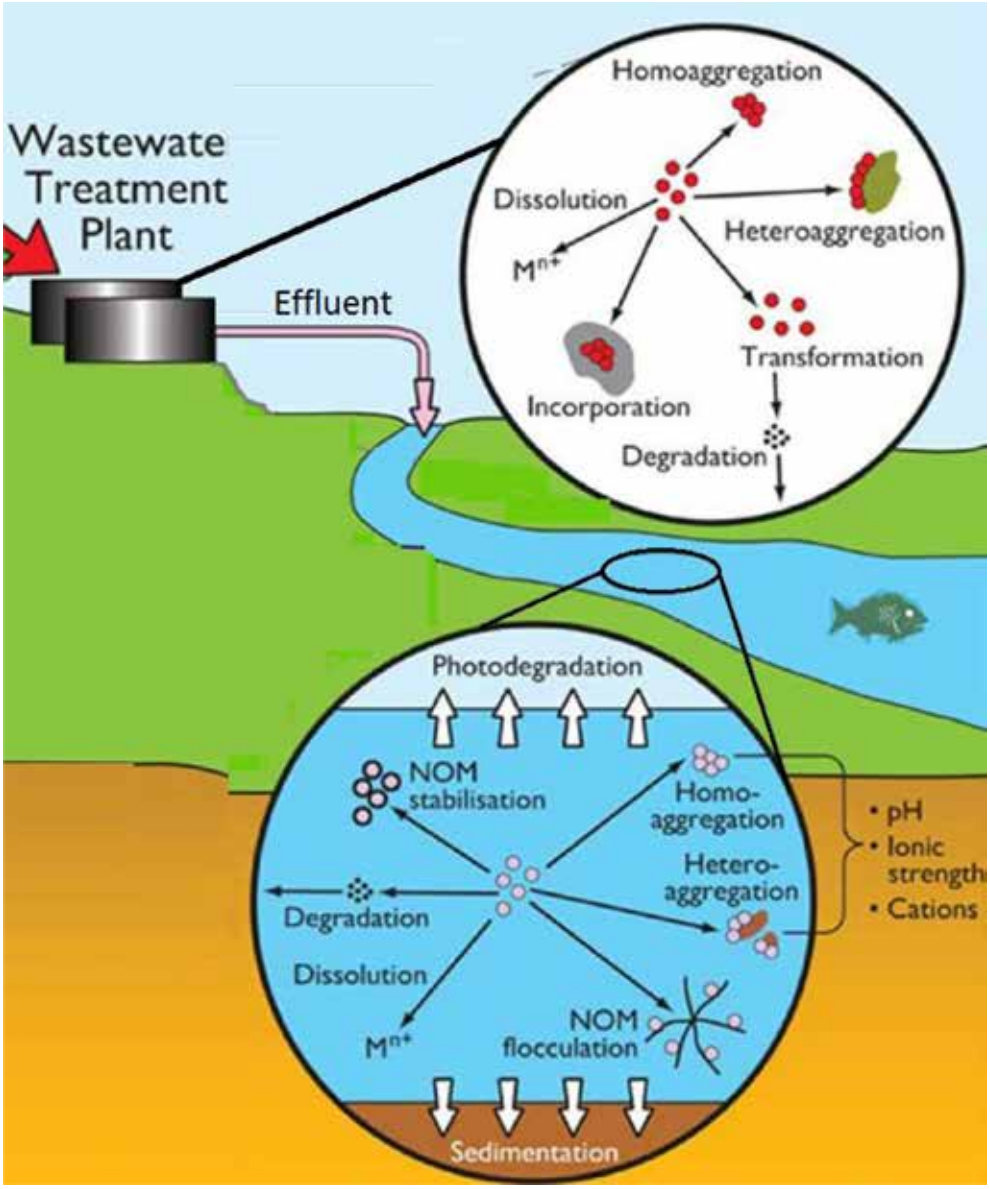

Figure 1. *Pathways and transformations of nanoparticles in the environment (Batley et al., 2012).*

nanoparticles into ionic zinc in pseudo feces on the one hand, but pack some of the cerium (IV) oxide nanoparticles into fecal pellets on the other hand (Montes et al., 2012). Thus, ENPs can be biotransformed into other forms and they can also be persistent for years and transported over a long distance since fecal pellets of some species, such as *Capitella teleta*, can be persistent for a long time (half-life of 5 to 33 years) (Gallagher and Keay, 1998). Cleveland et al. (2012) found an ex-

tensive leaching of Ag from Ag NPs in consumer products during the experimental period of 60 days in a modular estuarine mesocosm system. Ag NPs can exist as Ag^0 solids, free Ag^+ ions or its complexes, and surface-adsorbed Ag^+ (Liu and Hurt, 2010). In the aquatic environment, the presence of abundant inorganic ligands and organic ligands may form different complexes on Ag NPs surfaces. Lowry et al. (2012a) found that approximately 82% of the added Ag NPs in freshwater mesocosms were sulfidated into silver sulfides and silver sulfhydryl compounds. Recently, Buffet et al. (2012) showed that particles initially deposited on the sediment surface were transported progressively to deeper sediment layers (~5 cm).

Factors affecting the environmental fate of ENPs are numerous and diverse and we unfortunately know relatively little about how these complex processes and their interactions ultimately affect the distribution and fate of ENPs in the aquatic environment at this point in time.

1.2 Ecotoxicity of ENPs in the aquatic environment

the impact and potential toxicity of ENPs is one of the key issues in evaluating the safety of ENPs released into the aquatic environment. More and more studies have been published on the ecotoxicity of ENPs to aquatic organisms, especially through water exposure. Recently published studies are compared below, focusing on the influence of metal form, particle size and particle shape of ENPs on their toxicities to aquatic organisms.

Metal form. There is a current debate as to whether the source of Ag NPs toxicity is related to Ag NPs or Ag ions because they are co-occurred. A handful studies have tried to compare and discern the toxicity of the two metal species. For example, Miao et al. (2009) observed that the inhibition of photosynthetic activity and reduction in chlorophyll production in a marine diatom, *Thalassiosira weissflogii*, was a function of Ag ions released from Ag NPs. A linear correlation between Ag NPs toxicity and Ag ions was found in *Caenorhabditis elegans*, indicating that the dissolved Ag ions were the key parameter determining the toxicity of Ag NPs (Yang et al., 2011). He et al. (2012) observed a complete removal of the inhibitory effects of Ag NPs on a marine raphidophyte, *Chattonella marina*, by reducing bioavailable Ag ions with a strong Ag ion ligand (cysteine) in water exposures. Xiu et al. (2011) examined the toxicity of Ag NPs and Ag ions were

assessed for bacteria, *Escherichia coli*, under anaerobic conditions to prevent the oxidation of Ag NPs. The authors found that Ag ions were 20 times more toxic than Ag NPs (EC_{50}: 0.10 ± 0.01 mg L^{-1} for Ag ions vs. 2.04 ± 0.07 mg L^{-1} for Ag NPs). Navarro et al. (2008) also showed that Ag NPs alone had minimal toxicity to a freshwater alga, *Chlamydomonas reinhardtii*, even though it served mostly as a source of Ag ions.

However, other studies have suggested that Ag NPs toxicity cannot be explained solely by the release of Ag ions. Higher toxicity of Ag NPs than Ag ions was observed in the common grass, *Lolium multiflorum*, at a concentration up to 40 mg L^{-1} (Yin et al., 2011). Ag NPs caused abnormal growth with highly vacuolated and collapsed cortical cells and broken epidermis and root cap. Griffitt et al. (2008) concluded that Ag had an enhanced toxicity to freshwater cladocerans, *Ceriodaphnia dubia*, if present as Ag NPs compared to when present as the ionic form. Laban et al. (2010) also stated that the toxicity of Ag NPs to fathead minnow embryos, *Pimephales promelas*, was not attributed solely to the released Ag ions but rather the Ag NPs themselves. Both particles and Ag ions contributed to the toxicity of Ag NPs to human hepatoma HepG2 cells (Kawata et al., 2009).

Wang et al. (2012) compared systematically the contribution of released Ag ions to toxicity of Ag NPs in three species from different tropical levels (*Raphidocelis subcapitata*, *Chydorus sphaericus* and *Danio rerio*). It was found a positive relationship between toxicity contribution of released Ag ions and tropical levels, that *Raphidocelis subcapitata* (algae) was more sensitive to released Ag ions exposure; while *Danio rerio* (zebrafish embryo) was more sensitive to Ag NPs exposure (Wang et al., 2012). The toxicity contribution of Ag ions may relate to the amount of binding sites on cell membranes in organisms from different tropical level. For algae and bacterial, rapid uptake of Ag ions can be both via cation transporters (probably Cu^{+} transporters) pathway and via sulfate/thiosulfate transporters with presences of sulfate/thiosulfate (Odermatt and Solioz, 1995, Fortin and Campbell, 2000, Fortin and Campbell, 2001). For fish, uptake of Ag ions is mainly via Na^{+}/K^{+} transporter, which is competed with Na^{+} uptake (Wood et al., 2009). In addition, the relative toxicity of Ag NPs and Ag ions is made difficult in the presence of common ligands such as Cl^{-}, S^{2-} and PO_4^{3-} in test media, given these ligands can decrease the concentration of Ag ions and thus the toxicity (Xiu et al., 2011).
Particle size. In general, smaller ENPs are known to have higher abilities for cell invasion and greater probabilities to cause toxicities than larger ENPs (Oberdörster

et al., 2005). At sub-cellular level, size-dependent effects of ENPs are commonly observed. For instance, Ag NPs (< 5 nm) have a significant induction of intracellular reactive oxygen species (ROS) than larger particles in nitrifying bacteria (Choi and Hu, 2008). Ag NPs of 15 nm induce 10 times more ROS than 35 or 55 nm particles at a concentration of 50 μg L^{-1} in macrophages, and both 15 nm and 35 nm Ag NPs highly reduce mitochondrial function (reduction of the tetrazolium salt: $EC_{50\text{-}15,\ 35\ nm}$ < 45 μg L^{-1}, $EC_{50\text{-}55\ nm}$ > 75 μg L^{-1}) and membrane integrity (lactate dehydrogenase leakage: $EC_{50\text{-}15,\ 35\ nm}$ < 17 μg L^{-1}, $EC_{50\text{-}55\ nm}$ > 75 μg L^{-1}) (Carlson et al., 2008). Ten nm Ag NPs induce the heavy metal stress response gene and the production of metallothionein to a higher degree than 35 nm or 600-1600 nm particles in zebrafish (*Danio rerio*) embryo (Osborne et al., 2012). Ag NPs caused more DNA damage than micron-sized Ag particles in the polychaete, *Neries diversicolor* (Cong et al., 2011). Small Ag NPs inhibit the activity of Na^+/K^+-ATPase to a larger extend than Ag NPs in the earthworm, *Eisenia fetida* (Hu et al., 2012). At whole organism level, CuO NPs has been shown to cause increased mortality in the snail, *Potamopyrgus antipodarum* compared to micron sized CuO particles (Pang et al., 2012). Thus, size-dependent toxicity of ENPs would also be observed at the whole organism level based on differences of potential toxicity mechanisms caused by nanoparticle size. However, only a few studies address the effect of nanoparticle size on ENPs toxicity in aquatic organisms.

Particle shape. A few studies have examined the effect of NPs shape on toxicity. In one comparison, elongated titanium dioxide nanoparticles (44 nm × 1500 nm) caused approximately 10% mortality while spherical titanium dioxide nanoparticles (11 nm) caused greater than 50% mortality of *Escherichia. coli* (Simon-Deckers et al., 2009). Wang et al. (2008) found that gold nanoparticles in rods were more cytotoxic than 1.5 nm gold nanoparticles in spheres in human skin keratinocytes. Pal et al. (2007) found that truncated triangular Ag NPs inhibited bacteria (*E. coli*) more effectively than Ag NPs in spheres or rods. In addition, at the same concentration, needle-shaped hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) delayed embryo hatching ratio of zebrafish to a higher degree than rod-shaped hydroxyapatite after 72 h (Zhao et al., 2012). The toxic mechanism of ENPs in different shapes is still not fully understood and no toxicity studies of nanoparticle shape have been published in aquatic organisms so far.

These comparisons highlight the importance of considering deposit feeders and sediment exposure for assessing risks of ENPs. Clearly, the ecotoxicology of ENPs in sediment is still poorly understood and hence, a pressing need exists to collect toxic data for deposit feeders.

1.3 Uptake and depuration kinetics of ENPs in the aquatic environment

Assessing risks of ENPs in the aquatic environment requires understanding of the factors controlling their bioavailability and uptake and depuration kinetics to aquatic organisms.

Aquatic organisms have shown their ability to accumulate ENPs through both water and sediment exposure routes. For instance, ENPs are taken up in carp (*Cyprius carpio*), in fish and fish embryos (*D. rerio* and *P. promelas*), polychaetes (*N. diversicolor* also called *Hediste diversicolor*), bivalves (*Scrobicularia plana*, *Mytilus spp.,*), snails (*P. antipodarum*) and other taxa (Choi and Hu, 2008, Fabrega et al., 2011, Cong et al., 2011, Pang et al., 2012). Body burden of titanium in *Daphnia magna* increases with the exposure duration (Zhu et al., 2010). Zhao and Wang (2010) demonstrated that more than 70% of Ag NPs are accumulated in *D. magana* through digestion of algae-associated Ag NPs, which suggests the importance of dietary uptake route to ENPs bioaccumulation. *N. diversicolor* accumulates Ag from sediment amended with Ag NPs (Cong et al., 2011), and in addition, Garcia-Alonso et al. (2011) found that most of the accumulated Ag NPs were observed in the gut lumen and epithelium cells of *N. diversicolor* exposed to Ag NPs spiked sediment. This suggests that deposit feeders may also accumulated ENPs from sediment.

Little is known about form dependent uptake and depuration kinetics of NPs in deposit feeders. In a study by Khan et al. (2012), it was found that Ag accumulation in two marine snails *Peringia ulvae* and *Lymnaea stagnails* was dependent on metal form such that Ag NPs was accumulated to a lesser extend compared to Ag ions. The authors suggested that this result was due to a slower uptake of Ag NPs and a comparative, or even faster, efflux of Ag NPs compared to that of Ag ions. The efflux of accumulated Ag NPs consisted of two compartments elimination which a fast efflux of Ag NPs followed by a slow efflux of dissolved Ag ions (so

called 'Trojan horse' concept). Zhao and Wang (2012) found a slower uptake rate of Ag NPs at low exposure levels (1 and 4 μg/L) but a significant greater uptake rate of Ag NPs at high exposure level (500 μg/L) compared to uptake of Ag ions in *D. magna*. Similar results have been reported for zinc oxide nanoparticles and *D. magna*: zinc oxide nanoparticles were taken up significantly greater than zinc ions (Li and Wang, 2013). Hence, it appears that ENPs and the corresponding metal ions are likely taken up through different pathways in *D. magna*. In contrast, *D. magna* were able to depurate zinc accumulated from zinc oxide nanoparticles, which is suspected to be dissolved in the organisms and depurated through the same pathway as zinc ions (Li and Wang, 2013). Furthermore, although bioaccumulation of Ag is observed on the same level for Ag ions and Ag NPs in the polychaete, *N. diversicolor* after 10 days exposure (Cong et al., 2011), different detoxification pathways were suggested, given that accumulated silver nanoparticles were associated predominantly with inorganic granules, organelles and heat-denatured proteins while Ag ions were internalized in metallothionein fractions (García-Alonso et al., 2011). Thus, uptake and depuration kinetics of ENPs could be both metal form dependent and species specific.

Uptake of ENPs has been observed via endocytosis in mammalian cells (Conner and Schmid, 2003). Endocytosis is a process by which large molecules and particles are engulfed into cells. Endocytosis rates of single-walled carbon nanotubes (50 nm) and gold nanoparticles (50 nm) have been determined to be 10^{-3} min^{-1} and 10^{-6} min^{-1}, respectively (Wang et al., 2010). In addition, diffusion via chorion pore canals has been found to facilitate the uptake and depuration of small Ag NPs (5-46 nm) into zebrafish embryo cells (Lee et al., 2007). Elimination of accumulated nanoparticles has been documented via exocytosis (Jin et al., 2009). It is a process that a cell directs the contents of big molecules and particles out of the cell membrane and into the extracellular space. The rate constant via exocytosis has been determined to be 10^{-4} to 10^{-3} min^{-1} for poly(d,l-lactide-co-glycolide), single-wall carbon nanotubes, and gold nanoparticles across distinct cell lines (Jin et al., 2009). However, little is known about how different uptake and depuration pathways of ENPs will be reflected in their bioaccumulation and uptake and depuration kinetics in aquatic organisms. Therefore mechanistic studies are lack at this point in time to explain the differences in uptake rates for ENPs compared to other forms for aquatic organisms at this point in time.

1.4 Bioavailability and bioaccumulation of metal ions

There are extensive evidences that the bioavailability and toxicity of metal ions cannot be explained by either total metal concentration or dissolved aqueous metal concentration alone in aquatic environments. For instance, organic and inorganic metal complexes (i.e., formed with organic matter and inorganic ligands) have been recognized to be un-available and non-toxic to pelagic organisms such as fathead minnows (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*) (Di Toro et al., 2009). In the Biotic Ligand Model (BLM) (Paquin et al., 2000) which is based on a mechanical understanding of interactions of metal ions with biotic membranes, it is assumed that only the free metal ions can cause toxicity. This model has been successfully applied to predict Cu and Ag toxicity and bioavailability to organisms living in the water phase (Paquin et al., 2000, Santore et al., 2009). In marine sediments with low oxygen, metal ions are mainly associated with reactive sulfides, organic carbon and possibly other reactive ligands and surfaces (i.e., magnesium and iron oxides) (Tessier et al., 1979, Howard and Evans, 1993). Metals associated with acid volatile sulfides (AVS) are assumed to be unavailable for uptake and thus not toxic to aquatic organisms. By relating the concentration of metal to the concentration of AVS it is thus possible to estimate the potential bioavailable metal pool (called the AVS model). However, both models and their assumptions are not adequate to explain metal uptake for deposit feeders which can accumulate metals both in pore water and overlying water through the body surface and associated with sediment through diet over the gut epithelia (i.e., sediment ingestion of metals associated with AVS and organic matter fractions). Uptake from the sediment-associated pool has been shown to be the main uptake route in many deposit-feeding organisms including polychaetes (*C. teleta, Neanthes arenaceodentata, Nereis succinea*) and oligochaetes (*Lumbriculus variegatus*) (Selck et al., 1998, Lee et al., 2000, Wang et al., 1999, Camusso et al., 2012).

Several pathways have been suggested for uptake of trace metals through membranes of organisms, which includes (i) by passive diffusion of metal ions crossing the lipid membrane on a protein 'carrier', following a concentration gradient; (ii) by carrier protein mediated metal transporting across the membranes; (iii) via a metal ion channel with a hydrophilic path for diffusion; (iv) via endocytosis of metaliferous particles across membrane (Tessier et al., 1993). Once trace metals enters an organism, they are available to transformation resulting in elimination from or sequestration within the organism. Essential metals (i.e., copper and zinc)

include metals those are essential for metabolic processes, such as Cu, zinc, iron, silicon and so on. White and Rainbow (1985) estimated that 26.3 μg Cu g^{-1} and 34.5 μg zinc g^{-1} are needed for enzymes to fulfill their functions in mollusks and crustaceans. Non-essential metals are not needed for enzyme functions in aquatic organisms. Elimination and sequestration of the excess essential metals (i.e., more than 26.3 μg Cu g^{-1}) and nonessential metals may involve metallothionein/-like proteins, biomineralization and granules. Methallothioneins/-like proteins are a group of protein with high amount of cysteine and the thiol groups (–SH) of cysteine residues can bind particular metals. They can be induced by zinc, Cu, cadmium, mercury and Ag in invertebrates (Roesijadi, 1992). The strong binding of assimilated metals to thiol groups of methallothionein/-like proteins isolates them from sites if toxic action (Luoma and Rainbow, 2008). Biomineralization refers to sequestration and elimination of uptake metals by incorporating into relatively inert shells, exoskeleton and bones. Sequestrations in insoluble granules are usually associated with the mid gut, digestive gland, hepatopancreas, malpighian tubules and kidney of invertebrates (Roesijadi and Robinson, 1994). In addition, storage of granule associated metals is also important in the elimination of metals to the cell exterior (Howard et al., 1981).

Depuration refers to the removal of internalized trace metals resulting in a decreased concentration exposed organisms. Neither desorption of adsorbed metal nor defecation of un-assimilated metal in food can be considered as depuration (Luoma and Rainbow, 2008). The elimination pathway of metal involves transportation across the gills, exhalation, secretion from the intestinal mucosa, shedding of granules, molting and excretion via the kidney (Newman and Unger, 2003). The bound metals in exoskeleton can be eliminated by molting in chironomids (Krantzberg and Stokes, 1988). Insoluble granules exist broadly in aquatic organisms, on which bound metals may be discharged via exocytosis (fusion of intracellular vesicles to the cell exterior) or via cell lysis into the gut followed with elimination through urine (Newman and Unger, 2003). Depuration of accumulated trace metals can be predicted by depuration rate constants which are proportional rate constants of the total loss of trace metal from organisms. It usually described by first-order exponential decay model, which is directly proportional to the concentration in the organism, assuming no changes in body weight (Spacie and Hamelink, 1985, Luoma and Rainbow, 2008).

In summary, the bioavailability and bioaccumulation mechanisms for sediment-associated metal ions in aquatic organisms are relatively well understood. However, it is unclear to what extent these same mechanisms can be applied to ENPs.

1.5 Aims of the thesis

The overall aim of this thesis was to determine the toxicity and bioaccumulation of ENPs in two marine deposit feeders, *C. teleta* and *M. balthica* (Fig. 2). Both Ag NPs and CuO NPs were tested as models of ENPs. To determine whether metals added to the environment in nano-form differed from other forms of metals, both metal ions and bulk particles (referring to micron sized particles) were included in experiments for comparison with the corresponding ENPs. The toxicity of metals in different forms, nanoparticle sizes and shapes was investigated on mortality, growth and feeding rate of *C. teleta* and on mortality, burrowing activity, condition index and DNA damage of *M. balthica*. Bioaccumulation of Cu was characterized by an uptake and depuration kinetic method in both species, and accumulation parameters were compared among metal forms, metal nanoparticle sizes and shapes. Finally, released Ag ions were detected through DGT deployment in both seawater exposure and sediment exposure in order to evaluate the contribution of released Ag ions to potential bioavailability of Ag NPs to test species.

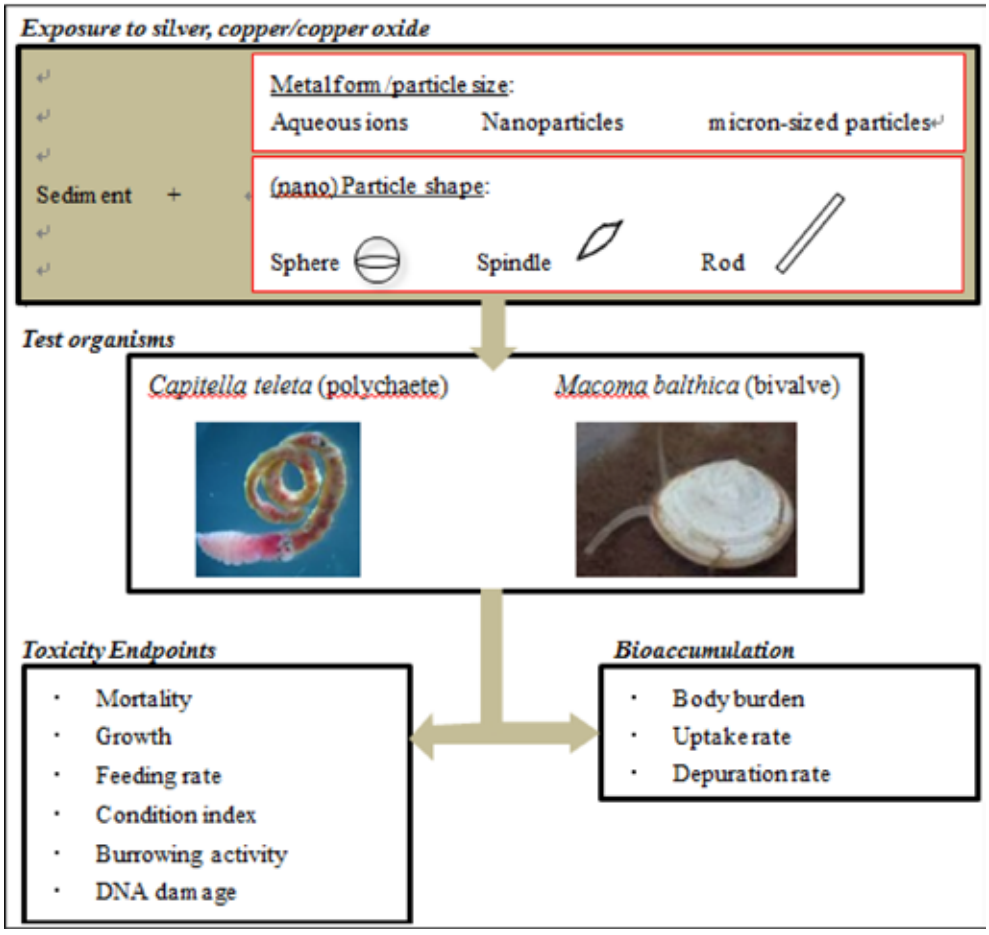

Figure 2. *Overall aims of this thesis.*

2. Materials and methods

2.1 Research overview

Paper I

Growing concerns on the necessity of characterization of ENPs used for toxicity evaluation were required to ensure reliable and reproducible results, as well as proper interpretation of toxicity results. Hence, the importance of fully characterizing ENPs before conducting ecotoxicological studies was addressed.

Paper II

In this study, toxic effects of sediment-associated aqueous Ag ions ($AgNO_3$), Ag NPs (20 nm, 40 nm, 100 nm) and micron Ag particles were tested on the marine deposit-feeding polychaete, *C. teleta*, using mortality, growth and feeding rate as endpoints. Ag body burdens were measured after 14 days. The worms were exposed to three nominal concentrations of 10, 50 and 100 μg/g dry weight sediment (dw sed).

Paper III

In this study, toxic effects of sediment-associated Cu in different forms (i.e., aqueous Cu ions ($CuCl_2$), 100 nm CuO NPs, micron CuO particles) and sediment-associated CuO NPs in different shapes (spheres, spindles and rods) were tested on *C. teleta*, using mortality and growth as endpoints. Uptake rate constants for Cu were evaluated by measuring worm body burdens over time on day 0, 1, 3, 4, 5 and 7 during exposure to spiked sediment. In addition, depuration rate constants for Cu were evaluated by measuring the decline in body burdens over time on day 2, 3, 4, 5 and 7 after worms were transferred to clean sediment. The worms were exposed to a nominal concentration of 200 μg/g dw sed.

Paper IV

In this study, toxic effects of both sediment-associated Ag and sediment-associated Cu in different forms (i.e., aqueous Ag ions ($AgNO_3$), 20 nm Ag NPs (PVP-coating), 80 nm Ag NPs (PVP-coating) and micron Ag particles; aqueous Cu ions ($CuCl_2$), 100 nm CuO NPs, micron CuO particles) were tested on the marine deposit-feeding mollusk, *Macoma balthica*, using mortality, condition index and burrowing behavior as endpoints. Uptake rate constants for Cu were evaluated by measuring body burdens over time on day 0, 3, 7, 11, 15, 23 and 35 during exposure to spiked sediment. In addition, depuration rate constants of Cu were evaluated by measuring the decline in body burdens over time for 15 days after clams were transferred to clean sediment. The clams were exposed to a nominal concentration of Ag or Cu at 200 μg/g dw sed.

Experimental notes (Released Ag ions from Ag NPs by DGT)

In this study, released ions from Ag NPs were tested by diffusive gradients in thin films (DGT) in different media, including water (aqueous Ag ions ($AgNO_3$), 100 nm Ag NPs-PVP coating) and sediment (aqueous Ag ions ($AgNO_3$), 20 nm Ag NPs, 40 nm Ag NPs and 100 nm Ag NPs). Sources and details of this experiment are listed in 'Experimental Setup'.

2.2 Deposit feeders

Deposit feeders are organisms that meet their nutritional requirements from the organic fraction of ingested sediment. Thus, they can be exposed to ENPs deposited on the sediment surface. Most sediment has an organic content of 5 % or less, and is therefore considered a relatively poor food source (Lopez and Levinton, 1987). Hence, deposit feeders have developed various strategies for dealing with this poor food source such as particle selection mechanisms (Self and Jumars, 1978, Lopez and Levinton, 1987). Particle selectivity is common in deposit feeders, and depends on both the organs that contact the particles and the accessibility of organisms to certain particles. Particle selectivity occurs on the basis of size, shape, specific gravity, surface texture and chemical composition of sediment particles (Whitlatch, 1974, Hylleberg and Gallucci, 1975, Self and Jumars, 1978, Taghon, 1982). In addition, digestion of food particles is also associated with specific digestion gland structure of some deposit feeders (such as mollusks) in which preferred food particles are selected internally for further digestion (Lopez and Levinton, 1987).

Both selective feeding and digestive processing of ingested particles may affect how deposit feeders obtain nutrition and accumulate pollutants. In this thesis, used species *C. teleta* and *M. balthica* differ in feeding behaviors and digestive physiologies which may influence the uptake and toxicity of ENPs.

2.2.1 *Capitella teleta*

C. teleta is a sibling species in the *Capitella capitata* complex which was first identified as *Capitella* sp. I by JR Grassle (personal communication). Subsequently, Blake et al. (2009) described the species morphologically and designated it as *C. teleta*. *C. teleta* has been described to feed in a conveyor-belt mode, which means that particles are ingested at depth and defecated on the sediment surface (Méndez et al., 1997). Selective feeding is believed to occur at the mouth, or 'caecum', followed by ingestion and retention of selected food-rich particles (Fig. 3). The particles that are selected preferentially for ingestion are 2–20 μm (Horng and Taghon, 1999). By studying the fecal pellets of the species using natural sediment or glass beads, it has been shown that particles larger than 40 μm can be ingested (Horng and Taghon, 1999, Madsen et al., 1997), but it is believed that smaller particles have high nutritional values due to their high surface area to volume ratio, and thus are preferred (Whitlatch and Weinberg, 1982, Lopez and Levinton, 1987). Selected particles pass through two digestive compartments, an anatomically-distinct foregut followed by a hindgut (Penry and Jumars, 1990). No particle sorting is believed to occur within the gut of *C. teleta*. The gut fluid is enriched in enzymes (i.e., protease, esterase, lipase, glycosidase), surfactant micelles, dissolved amino acids and dissolved lipids based on analysis of the gut extract from several deposit feeding polychaetes (Mayer et al., 1997). In regards to pollutant uptake,

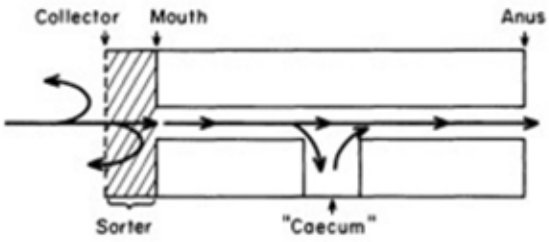

Figure 3. *Schematic representation of selective feeding of a deposit feeder (Lopez and Levinton, 1987).*

surfactant micelles are thought to be responsible for the dissolution of sediment associated hydrophobic contaminants (i.e., polycyclic aromatic hydrocarbons) in the gut of a deposit-feeder (Voparil et al., 2004). Amino acids are responsible for the dissolution of sediment associated metals (i.e., Cu) in the species guts (Chen and Mayer, 1998). However, the specific composition of the gut fluid of *C. teleta* has not been analyzed or identified so far.

2.2.2 *Macoma balthica*

M. balthica is a tellinid bivalve. It burrows below the sediment surface and feeds upon particles of small size, low specific gravity, and high organic content (Hylleberg and Gallucci, 1975) depositing on the sediment surface through a long, muscular siphon. It has been determined to be a facultative feeder, switching between suspension feeding and deposit feeding modes, depending on the food quality and quantity in the water phase (Lin and Hines, 1994), such that when no suspended food is available in the water phase, *M. balthica* feeds on sediment deposit. Once food material enters the siphon, the initial processing involves a physical sorting on the gills that is thought to be based on particle size, density, or perhaps nutrient quality (Bayne and Newell, 1983). Larger, less desirable particles are rejected as pseudo feces through the incurrent siphon or mantle, and are often bound by mucus. Small, desired particles are ferried to the mouth and stomach where further sorting occurs and digestion begins. Solutes and colloidal organic material (and associated pollutants) may cross the membranes of the stomach and intestine after digestive enzymes degrade labile organic matter (Reid and Räucher, 1972, Bayne et al., 1989, Widdows et al., 1979). This is called 'intestine digestion' (Widdows et al., 1979). Finer particulate material is further sorted from these solutes while in the stomach and sent to the tubules of the digestive gland. Here digestive cells phagocytize the particles and digest them intracellularly (Luoma, 1983). This is called 'glandular digestion' (Widdows et al., 1979). However, particle sorting during digestion is not only a matter of particle size, but also complicated by other factors such as the chemical properties of the different particle types (Brillant and MacDonald, 2000). Decho and Luoma (1991) found the same gut passage time of 15 μm latex beads as that of bacteria (which are considerably smaller) in *M. balthica*. The gut fluid of *M. balthica* has not been extracted and analyzed directly yet. Based on results of Weston and Maruya (2009), bioaccumulation of metals and polycyclic aromatic hydrocarbons in *Macoma nasuta* was

less than dissolved metals or polycyclic aromatic hydrocarbons in the gut fluid extracted from the polychaete, *Arenicola brasiliensis*. This suggests that pollutants would have different concentrations or activities in the digestive fluids of different taxa (Mayer et al., 2009, Weston and Maruya, 2009). This may also be the case for ENPs in mollusks.

2.3 Experimental preparation

2.3.1 *Capitella teleta*

C. teleta used in this thesis was derived from laboratory cultures of worms that were originally collected in Setauket Harbor, New York, USA and identified as *Capitella* sp. I by JR Grassle (pers. com.). Worms were cultured in sieved, field-collected sediment (2 to 5 cm layer, grain size < 250 μm) and seawater (31‰) at 17°C. In Paper II, several weeks before exposure, brood tubes (containing females with eggs) were removed from the culture, and eggs were allowed to hatch in seawater. Afterwards, free-swimming larvae were transferred into a glass beaker with clean sediment, seawater (31‰) and an air pump until they were approximately 14 d old. In both Paper II and III, worms used were sieved out of the lab culture and transferred into clean seawater (31‰) to empty their guts for 24 h before exposure.

2.3.2 *Macoma balthica*

M. balthica was collected from different sites of Isefjorden at different times since it was difficult to collect enough animals for experimental purpose (Paper IV). Before exposure, clams were kept in clean seawater (16‰) for two weeks without food. For Cu exposure, clams were grouped into small clams (8-11 mm) and large clams (11-14 mm) for the uptake experiment and depuration experiment, respectively, since clam size may influence metal bioaccumulation (Lee et al. 1998).

2.3.3 *Sediment preparation*

Sediment used in all experiments was collected from Isefjorden (55°40'N, 11°47'E, Munkholm, Denmark). In the field, the top few centimeters of the sediment

surface were scraped off and sieved to less than 1 mm with seawater. After sediment was transferred to the lab, raw sediment was sieved (*C. teleta*: 125 µm and *M. balthica*: 500 µm) in distilled water to kill meiofauna. Sieved sediment was rinsed twice with seawater (16‰) and frozen at -20˚C for several weeks prior to sediment spiking. Prior to spiking, sieved sediment was thawed at room temperature. Organic matter content of sieved sediment was determined from loss of weight on ignition at 550 °C for 6 h.

Sediments were spiked by adding known volumes of stock solutions of metal ions (i.e., $AgNO_3$, $CuCl_2$) or known volumes of stock suspensions of nanoparticles and micron-sized particles in order to obtain nominal concentrations for different experiments. Stock solutions of metal ions were prepared by dissolving metal salts in MilliQ water. Stock suspensions of ENPs supplied were directly added into clean without preparation. For Ag particles supplied in powder, stock suspensions (i.e., some nanoparticles and micron-sized particles) were prepared in MilliQ water (18.2 Ω, Millipore) and ultrasonicated (80 W, 45 kHz) in a water bath (Ultrasonic Bath VWR, Lutterworth, UK) for 15 min followed by a 15 min pause. This process was repeated 4 times. Stock suspensions of Cu particles supplied in powder were prepared in MilliQ water without ultrasonification. Before spiking sediment, stock suspensions (i.e., both prepared ourselves as mentioned and provided from project partners) were shaken intensively. For control sediment, without addition of stock solutions or stock suspensions, the same volumes of MilliQ water were added into clean sediment. Spiked sediment and clean sediment were homogenized by shaking for 24 h on a shaking table or by metal spoons, which depended on the amounts of sediment needed for different exposures. Overlying water phases of spiked sediment or clean sediment were removed and metal concentrations in spiked sediment or clean sediment were measured by flame atomic absorption spectrometry (AAS, SpectrAA-220, Varian, and Austrilia).

Details of the experimental setup and design can be found in Papers II, III and IV. All beakers used for exposures were acid washed (Fig. 4).

Figure 4. *Experimental setups. Capitella teleta, Paper II (Left); Capitella teleta, Paper III (Middle) and Macoma balthica, Paper IV (Right).*

2.4 Experimental notes (released Ag ions from Ag NPs by DGT)

DGT have been developed to measure metal ions and speciation in different matrices (i.e., water, soil and sediment) to predict the bioavailable metal pools in each matrix. The devices consist of two layers of polyacrylamide hydrogel, held in a rigid plastic frame, with a window to allow exposure to the matrix being studied (Fig. 5) (Davison et al., 2007). The window is usually covered by a filter membrane with a mesh size of 0.45 μm to prevent mechanical damage of large particles. The gel layer immediately behind the filter controls the flux of measured metal speciation from the matrix to a binding agent, which is supported in a second layer of gel which Chelex 100 is used for trace metals binding. DGT locally perturbs the chemistry of the matrix in which it is deployed, by removing dissolved components from the dissolving phase in contact with the exposure window. Dissolved metal ions/species diffuse through the filter and gel layer, until they reach the binding layer, where they are immobilized, reducing the effective concentration to zero. This results in a concentration gradient through the diffusive gel layer. The steepness of the gradient is governed by the concentration of solute at the surface of the device, and the sum of the diffusion layer thickness (Δg) and diffusive boundary layer thickness (δ). In the boundary layer, transport of metal ions/species is solely by molecular diffusion and thickness of this layer (δ) is negligible compared to Δg (DGT Research, http://www.dgtresearch.com/). The mass of solute (M) collected per unit area (A, cm^2) in the binding gel, divided by the deployment time (t), gives the time-averaged flux (F) of solute through the diffusion gel with typical units of mol cm^{-2} s^{-1}. In addition, according to Fick's First Law of Diffusion, F can also be determined by the measured flux to the solute concentration in the pore water adjacent to the exposure window of DGT (Eq. 1),

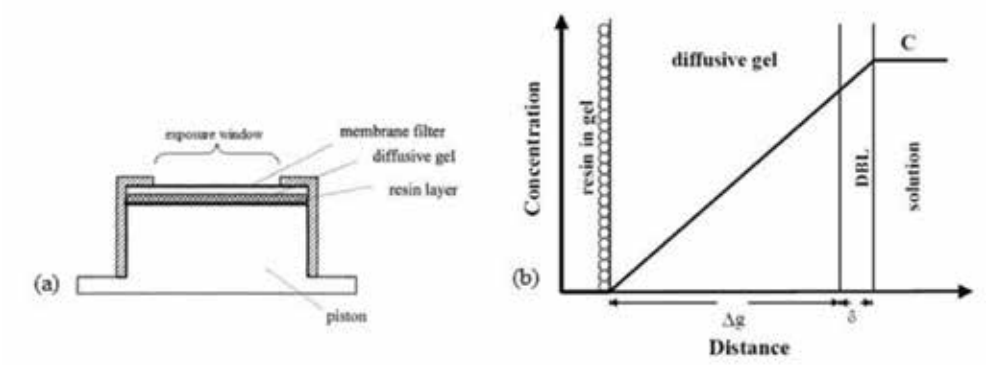

Figure 5. *Schematic cross-section (a) through a DGT device and (b) a concentration gradient in contact with solution, showing the steady-state concentration gradient. The diffusive layer is shown as a single layer of gel, but it can include a gel layer and filter. The thickness of the diffusive boundary layer (DBL) in solution depends on the rate of water movement (DGT Research, http://www.dgtresearch.com/).*

$$M/(A \times t) = F = D \times C_{DGT}/\Delta g \qquad \text{Equation 1}$$

By rearrangement of Equation 1, DGT measured concentration (C_{DGT}, μg/L) at its window surface can be calculated using Equation 2.

$$C_{DGT} = F \times \Delta g / D = M \times \Delta g / (D \times A \times t) \qquad \text{Equation 2}$$

Where D ($cm^2\ s^{-1}$) is the diffusion coefficient in the diffusion layer and by combining with Equation 1 enables calculation of C_{DGT}.

The mass of solute (M) is calculated as total solute amount binding on resin layer according to Equation 3.

$$M = C_e \times (V_g + V_e) / f_e \qquad \text{Equation 3}$$

Where C_e ($\mu g\ L^{-1}$) is metal concentration measured in 1M HNO_3 by AAS, V_g (ml) is volume of resin gel (0.15 ml typically), V_e (ml) is volume of HNO_3 used and f_e is the elution factor for each metal, typically 0.8 by using 1M HNO_3 (DGT research, http://www.dgtresearch.com/).

Deployment of DGT in spiked media with Ag in various forms

Quantifying the released metal ions from ENPs is of importance for both hazard identification and determining exposure levels during environmental risk assess-

ment of ENPs. Loaded DGT units (DGT Research, Lancaster University, UK) with filters and gels were deployed in both seawater and marine sediment spiked with Ag in various forms (aqueous Ag ions, Ag NPs and micron-sized Ag particles) for a defined time and temperature. The same spiking methods were used for Ag in various forms as those mentioned above (Paper II).

Water deployment. The window side of a DGT unit was totally immersed in seawater (31‰) spiked with aqueous Ag ions, Ag NPs (100 nm, PVP coating, Sigma-Aldrich) for 14 days. The average temperature was 21±2ºC.

Sediment deployment. The window side of a DGT unit was submerged into Ag spiked sediment (Ag forms: aqueous Ag ions, Ag NPs and micron-sized Ag particles) to a depth of 0.5 cm. The average temperature was 21±2ºC. After deployment, DGT units were taken out of spiked sediment, and the last hydrogel layer was rinsed with 1 ml 1M HNO_3 for 24 h. The acid extract (C_e) was measured on flam AAS and used for M and C_{DGT} calculations (Eq. 3).

3. Main results and discussion

3.1 The importance of characterizing ENPs before conducting toxicity tests (Paper I)

A consensus is growing on the necessity of proper and accurate characterization of NPs in environmental media to ensure that reliable and reproducible toxicity tests are performed. Although toxicity of ENPs has been measured, limited information is available on their mechanisms of toxicity. In order to compare different studies, there is a need to know detailed features of tested ENPs because of their high reactivity in the environment. Otherwise, toxicity test results may be questioned due to unknown variability in ENPs' properties and behaviors (Warheit, 2008).

The behavior of ENPs can be influenced by inherent and environmental factors, such as inherent properties of nanoparticle size, shape, surface area, surface charge, crystal structure, coating, solubility/dissolution and environmental properties of pH, ionic strength, salinity, organic matter and so on in dispersed media. Characterization of ENPs and test media is time consuming, exhaustive and costly. Therefore, some principle characteristics deserve quantification before conducting toxicity tests, which are size, shape, dispersion state, chemical composition, and surface charge (Powers et al. 2006). In this thesis, tested ENPs have been characterized regarding size, dispersion state, shape, surface charge, and crystal structure (Paper II, III and IV). The prepared MilliQ suspensions were the same as those used for sediment spiking in all experiments.

Ideally, characterizations should be conducted of the test media (i.e., seawater or sediment) because tested ENPs may undergo changes of size, dispersion state and surface coating after being transferred from MilliQ water into test media. However, currently there are methodological limitations to characterizing ENPs in sediment. As an absolute minimum, and until appropriate methods are developed,

full characterization of tested ENPs should be conducted in stock solutions used for spiking test media before conducting toxicity tests.

3.2 Uptake and Depuration Kinetics of Silver and Copper/Copper Oxide (Paper II, III & IV)

In Papers II, III and IV, Ag and Cu in all forms, nanoparticle in all sizes and shapes were bioaccumulated in the tested species, *C. teleta* and *M. balthica*. However, bioaccumulation, uptake and depuration kinetics as a function of metal form, nanoparticle sizes and shapes were differed between species (i.e., compare *C. teleta* (Paper II & III) with *M. balthica* (Paper IV)).

Capitella teleta. The bioaccumulation of both Ag and Cu was not form- or nanoparticle size- dependent as determined by the specific body burdens of Ag and Cu in exposed worms (Papers II & III). In addition, there were no form- or nanoparticle size dependent differences in uptake rate constants or depuration rate constants of Cu (Paper III). But nanoparticle shape was a matter to bioaccumulation, uptake and depuration kinetics of CuO nanoparticles in *C. teleta* (Paper III).

The bioaccumulation and the net uptake rate of Cu was a consequence of uptake from all environmental sources (i.e., water and sediment) and depuration in *C. teleta*. That positive net uptake rate constants indicated that Cu in all forms and CuO NPs in all sizes and shapes in Paper III have the potential for bioaccumulation in *C. teleta*. No dissolution of similar CuO NPs as used (i.e., 100 nm from Intrinsiq Materials Limited) has been detected in marine sediment by diffusive gradients in thin films (Buffet et al., 2011). Otherwise, the net uptake rate of CuO particles would be overestimated in Paper III if Cu ions were released from CuO NPs.

The observed similar net uptake rates of Cu administered in different forms suggest that Cu uptake from CuO particles was independent on Cu form. For the different CuO NP shapes, uptake rate constants was higher from sediment amended with spindles and rods compared to spheres. Uptake of Cu ions involves transportation across the cell membrane through available protein carriers (Luoma, 1983); while uptake of NPs occurs via endocytosis (Conner and Schmid, 2003). Endocytosis rates of single-walled carbon nanotubes (50 nm) and gold nanoparticles (50 nm)

have been determined to be 10^{-3} min^{-1} (equal to 1.440 d^{-1}) and 10^{-6} min^{-1} (equal to 0.001 d^{-1}), respectively (Wang et al., 2010), which is comparable to the rates measured in the present study (i.e., 0.104 d^{-1} for 100 nm CuO NPs; 0.192 d^{-1} for micron CuO paticles; 0.082 d^{-1} for spheres; 0.394 for spindles and 0.283 d^{-1} for rods; Paper III). In addition, different endocytotic pathways and uptake rates have been displayed in direct internalization of nanoparticles in different cells. Positive charged nanoparticles are preferentially internalized into in HeLa (cervical cancer cell line) cells by the clathrin-mediated endocytotic pathway at a higher uptake rate than negative charged nanoparticles (Harush-Frenkel et al., 2007). Uptake rate of negative charged cerium oxide nanoparticles was found higher than positive charged nanoparticles in adenocarcinoma lung cells (Patil et al., 2007). In a study of cellular internalization pathways of nanoparticles by Gratton et al. (2008) found that lager particles of 50-100 nm were internalized through the caveolae-mediated endocytotic pathway. Thus, different uptake pathways and cell lines may be involved in the internalization of CuO NPs and micron CuO particles in Paper III.

The effect of particle shape has not been fully understood on the uptake of NPs yet. Gold spheres (14 nm and 74 nm) were taken up approximately 3 times more than gold rods (14 nm × 74 nm) into HeLa cells after 6 hours (Chithrani et al., 2006). On the contrary, Gratton et al. (2008) found a higher uptake rate of poly-organic nanoparticles in rods (150 nm × 450 nm and 100 nm × 300 nm and), followed by cubes (200 nm × 200 nm) and spheres (1 μm, 2 μm, 3 μm and 5 μm) in HeLa cells. It was suspected that more receptors on cell surface interacted with the long axis of rods facilitating their uptake (Hutter et al., 2010). This may also be the case for the uptake of CuO NPs in spindles, rods and spheres in Paper III that the orientation of the long axis facilitates their internalization into *C. teleta*.

After 7 days of depuration, the body burden of Cu was decreased to the same background level as the control worms regardless of Cu treatment. These results suggest that C. *teleta* can eliminated Cu regardless of Cu form or nanoparticle shape taken up. Depuration of accumulated Cu ions involves that intracellular Cu is sequestered in cytosolic proteins (i.e., metallothioneins/-like proteins) and transported to lysosomes for degradation into insoluble residual bodies followed by excretion (Amiard et al., 2006, Wang and Rainbow, 2010). It is not clear what depuration mechanisms are involved in depuration of Cu NPs. Accumulated Cu may be eliminated both as particles and ions. However, it is not possible to

distinguish between the two using the one-compartment model which was used in Paper III. Nevertheless, the high elimination rate constants (k_ds) of spindles and rods suggest that they are eliminated mostly as particles. The 'Trojan horse' concept has, in addition to the toxicity aspect, also been proposed for the elimination of ENPs, in which the discrete metal ions delivered by ENPs into tissues are transported and eliminated through the existing cellular processes (Park et al., 2010). For instance, Ag accumulated from Ag NPs was eliminated in two compartments, that Ag NPs were eliminated at a higher rate in first 4 days followed by elimination of discrete Ag ions at a slower rate in deposit feeding invertebrate, *P. ulvae* (Khan et al., 2012). This may also be the case for depuration of Ag NPs because the presence of surfactants and proteinaceous materials in digestive fluid may facilitate Ag ion released from accumulated Ag NPs in polychaete, *C. teleta*. It has been reported that the desorption of Cu ions from ingested sediment were increased in the presence of histidine, proteinaceous materials (studied on its surrogate, bovine serum albumin) and sodium taurocholate (present in surfactants micelles) in digestive fluid of polychaetes (Zhong et al., 2012, Pettibone et al., 2008, Jones and Turner, 2010). In addition, Golobic et al. (2012) found that 99% Cu accumulated from food (common hazel leaves) spiked with Cu NPs was present as Cu ions in the isopod, *Porcellio scaber*. Therefore, depuration of released metal ions can be eliminated from the depuration of ENPs in *C. teleta*. However, more studies should be undertaken to understand the dissolution of CuO particles in the gut of *C. teleta* and its role in bioavailability of ENPs.

Macoma balthcia. Bioaccumulation of both silver and copper in soft tissue was form- and particle size- dependent in this species (Paper IV). There were effects of both form and particle size on net uptake rates and depuration rates of Cu. For aqueous Cu ions, bioaccumulation was characterized by a high (net) uptake rate and a relatively low depuration rate. For particulate CuO, bioaccumulation was characterized by positive (net) uptake rates but no depuration. CuO NPs were taken up faster than micron CuO particles.

In Paper IV, increased body burden of Ag and Cu suggested metals in all forms were bioaccumulated in *M. balthcia* after 35 days of exposure. Ratios of body burdens to sediment concentrations of metals were approximately 2 for aqueous Ag ions (clam size of 11.0 ± 2.0 mm), approximately 12 for aqueous Cu ions in clams of 9.6 (± 1.4) mm and 6 for aqueous Cu ions in clams of 12.0 (± 1.0) mm. In the field, the bioaccumulation factor of *M. balthica* can vary from 0.04

to 60 for Ag and from 0.05 to 8 for Cu based on metal concentrations in soft tissue (Luoma et al., 1983, Luoma et al., 1991). I did not measure whether body burdens reached steady state in Paper IV and it is likely they did not. So the body burden of Ag and Cu may have increase to even higher levels than I observed. However, the body burden of both aqueous ions (i.e., Cu and Ag) was extreme high compared to body burdens of clams from unpolluted sites (Cu: up to 300 μg/g dw tissue; Ag: up to 100 μg/g dw tissue from Luoma et al. (1991)) which may be related to a very high exposure concentration in the experiment (Cu: up to 200 μg/g dw sed; Ag: up to 200 μg/g dw sed) compared to unpolluted sites (Cu: up to 100 μg/g dw sed; Ag: up to 2.5 μg/g dw sed in Luoma et al. (1991)). Such high body burdens of Ag and Cu without detected toxicity, especially in small clams (Experiment 2, Paper IV), may be due to the strong adaptive ability of the species as a consequence of metal detoxification. Adult bivalves seem to be able to detoxify the accumulated Ag and Cu ions by binding them to metallothionein/-like proteins and by enclosing them in sulfide-rich granules and basal membranes of cells (George et al., 1986, Johansson et al., 1986). In addition, extreme high Cu body burden in small clams can be detoxified by inducing metallothionein/-like proteins at higher concentration as evidence from observed negative relationship between organism size and metallothionein/-like proteins concentration (Bordin et al., 1997, Mouneyrac et al., 2000).

Body burdens of Cu from Cu NPs and micron CuO particles were also higher in small clams than in big clams in Paper IV. Strong and Luoma (Strong and Luoma, 1981) found size dependent uptake rate in some populations of *M. balthica* from San Francisco Bay. It was also confirmed by Lee et al. (1998), that weight-specific influx rate of cadmium were negatively related to the size of *M. balthica*. This may be the case for big clams with a lower uptake rate than small clams in Paper IV. However, effects of organism size on bioaccumulation of CuO in different forms need to be further investigated in other experiments.

A form dependent net uptake of Cu and Ag was observed in Paper IV, such that there was a trend that k_u decreased with increasing particle size. This may be related to the particle sorting commonly existing in bivalves. During exposure to glass wool (silicon dioxides, 3-7 μm length and 0.18-1 μm), particle sorting has been observed in the bivalve, *Mytilus edulis* where larger fibrils were found in gill epithelial cells at high amounts, and only small and fine particles (up to 200 nm) entered the primary tubules after 12 h and appeared finally in the secondary

tubules after 24 h (Koehler et al., 2008). In addition, a longer gut retention time of polystyrene nanoparticles than 10 μm beads indicated that most nanoparticles were taken up in the digestive gland via endocytosis (Ward and Kach, 2009). No dissolution of CuO NPs has been detected by Buffet et al. (2011) in marine sediment (15‰) amended with CuO NPs from the same producer as used in Paper IV. Otherwise, the k_us of CuO particles reported here would be overestimated if dissolution occurred in the sediment. Low net uptake rate of CuO NPs than aqueous Cu ions suggested form dependent uptake of Cu in *M. balthica*.

As described for *C. teleta*, different uptake pathways and cellular lines may be involve in the internalization of positively charged CuO NPs and negatively charged Micron CuO particles in Paper IV. But it should be confirmed further by the characterization of CuO particles in sediment compartment since many factors may affect the behaviour of ENPs (i.e., cations, organic matters and pH).

In Paper IV, the k_d for Aqueous Cu ions (7.4% d^{-1}) was close to a typical depuration rate constant of 5% d^{-1}, which was described best by a one-compartment model (cited in Griscom et al. (2002)). As mentioned above, accumulated Cu ions are sequestered in cytosolic proteins (i.e., metallothionein/-like proteins) followed by transportation to lysosomes for degradation into insoluble residual bodies, which are excreted out of organisms (Amiard et al., 2006, Wang and Rainbow, 2010). There was no significant decrease of accumulated Cu from CuO NPs in Paper IV.

Difference in loss dynamics suggested that the form of Cu accumulated in *M. balthica* from sediment associated CuO NPs was probably not Cu ions. The depuration mechanisms of accumulated CuO NPs were not very clear. However, a different detoxification pathway of CuO NPs was implied by a significant association of Ag NPs with organelles and metal-rich granules in polychaete, *N. diversicolor* (García-Alonso et al., 2011). Compartmentalization of accumulated CuO NPs should be studied further in *M. balthica* to understand its strategies for ENPs detoxification.

Incorporation of metals into bivalve shells commonly involves two sources: 1) metals accumulated in the soft tissue of bivalves may be transferred to the shell during shell deposition from mantle tissue (Jodrey, 1953), and 2) adsorption onto shell surface from the surrounding external environment. Cu accumulated in shells of *S. plana* was shown to be a result of passive absorption of Cu in seawater (Bryan

and Uysal, 1978). Since no growth of shells was occurred during exposure in the present study, the bioaccumulation of Cu in the shells (including control clams) is thus most likely due to the absorption of different Cu forms from the sediments onto shell surfaces. As such, this form of bioaccumulation likely has little impact on the clam itself. However, it may still be of importance for bioavailability to predators of clams and thus to trophic transfer of metals including ENPs.

3.3 Toxicity of silver and copper/copper oxide (Paper II, III & IV)

Both Ag and Cu toxicity was not detected in both species on feeding rate, burrowing activity, growth, condition index and DNA damage. The only acute toxicity was observed as an approximately 23% mortality of *C. teleta* caused by Cu exposure (Paper II), which was independent of metal form, nanoparticle size and nanoparticle shape. Although Cu is an essential element to support physiological requirements in organisms, it may cause damages to aquatic organism by interfering with osmoregulation, inhibiting respiratory enzymes and reducing the activities of regulatory enzymes of ATP synthesizing pathways at high levels (Hodson et al., 1979, Hubschman, 1967, Hansen et al., 1992). The mortality of the shore crab, *Carcinus maenas* exposed to Cu was found to be caused by tissue hypoxia associated with reduced enzyme metabolisms (Hansen et al., 1992). Although different toxicity mechanisms have been proposed for ENPs, especially Ag NPs, no toxicity of Ag NPs was observed in my studies for *C. teleta* or *M. balthica*.

The lack of toxic effects observed for the other endpoints could be due to a relatively high adaptation and detoxification ability of the tested species to metal exposures. Metal exposures can induce detoxification processes, such as formation of metal binding proteins (i.e., metallothioneins/-like proteins), metal-containing extracellular granules, mineralized lysosomes and excretion of metals (Bryan and Hummerstone, 1977, Ng et al., 2008, Fernandez and Jones, 1989). It has been demonstrated that both Ag and Cu can induce metallothioneins/-like proteins in *M. balthica* (Bordin et al., 1997, Boisson et al., 1998). Moreover, it has been identified in many species, such as polychaete, *Alvinella pompejana* and *N. arenaceodentata* (Roesijadi and Fowler, 1991). Although metallothioneins/-like proteins has been demonstrated not to be responsible for detoxification of Ag NPs in the polychaete, *N. diversicolor* (García-Alonso et al., 2011), other pathways may exist

for detoxification of accumulated Ag NPs and CuO NPs in tested species, which cannot be answered through this thesis.

3.4 Released silver ions from silver ENPs (Experimental notes)

Water deployment. The Ag concentration measured was 2.59 and 1.41 mg/L in seawater spiked with aqueous Ag ions and 100 nm Ag NPs, respectively. The C_{DGT} was 4.2 ± 0.2 µg/L in water spiked with aqueous Ag ions and 0.1 ± 0.1 µg/L in water spiked with Ag NPs after 14-days deployment, which accounted for 2% and < 0.01% of the amount of Ag added to the water phase, respectively. Since formation of massive precipitation and complexation of Ag with inorganic ligands in seawater (i.e., Cl^-), the availability of free Ag ions for uptake by DGT accounted for only a tiny part of the Ag measured in the aqueous Ag ion treatment. That less than 0.01% of the total Ag taken up by the DGT in the Ag NPs treatment, indicates that there was very limited dissolution of Ag NPs in seawater (31‰).

Sediment deployment. The measured Ag concentrations in sediment on day 0 were reported in Paper II. In brief, no significant difference was detected in Ag concentrations in spiked sediment with the same nominal concentration used for DGT deployment (Fig. 6). After 14-days deployment in spiked sediment, no interactions between Ag form and nominal concentration were detected for the DGT parameters M_{Ag} or C_{DGT} (Blocked two-way ANOVA, $p = 0.730$; C_{DGT} not shown; 20 nm treatment omitted). A reduced model was run without the interaction term. However, neither nominal concentration nor Ag form affected M_{Ag} significantly (Blocked one-way ANOVA, $p = 0.768$ for nominal concentration and $p = 0.934$ for Ag form). The average M_{Ag} was 36.5 (± 7.4) ng and the average C_{DGT} was 82.8 (± 12.5) µg/L for data pooled across nominal concentrations and Ag forms, corresponding to less than 0.02% of the total sediment Ag pool which suggests that an extreme low availability of free Ag ions in sediment exposures of Ag NPs.

DGT consists of three membranes with a pore size of 0.45 µm for the first filter layer, and hydrogel and resin gel have been assumed to have pore sizes not exceeding 10 nm (Zhang and Davison, 1999). Van der Veeken et al. (2008) found that 129 nm spherical carboxylated latex particles associated with lead ions (Pb^{2+}) on particle surfaces can pass the hydrogel layer and bind on the Chelex-100 pre-

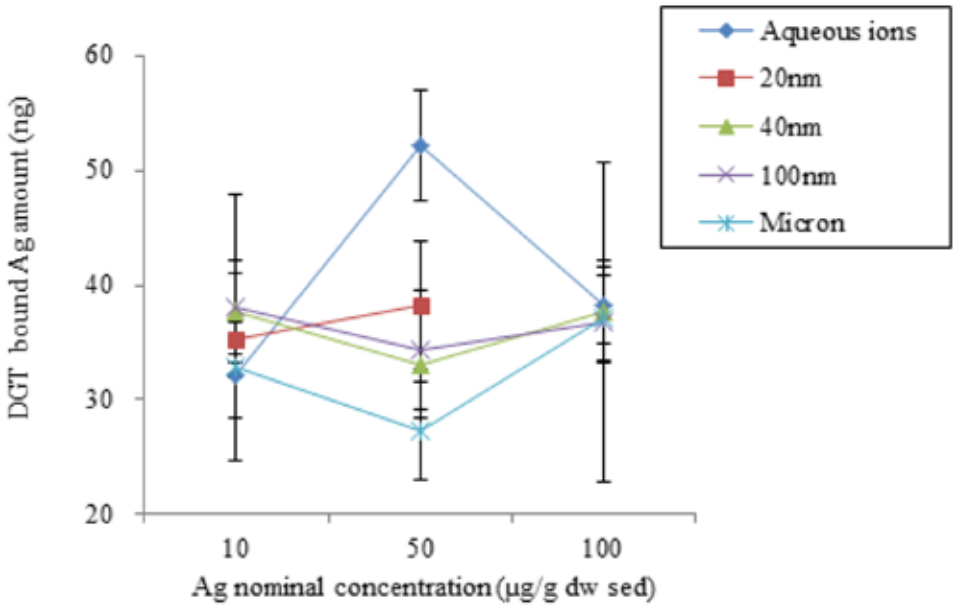

Figure 6. *DGT bound Ag (M_{Ag}) in sediment spiked with Ag in various forms for 14 d deployment.*

pared resin gel. As showed in Paper II, the hydrodynamic size distribution of our Ag NPs were 21.2 nm, 44.3 nm and 97.4 nm on average for 20 nm, 40 nm and 100 nm Ag NPs characterized in maltose solution, respectively. The micron-sized particles used here were polydispersed with a wide size range from 8 nm to 3000 nm (Cong et al., 2011). These characterization data may explain the similar M_{Ag} for different Ag forms to some extent, but it was not possible to characterize Ag NPs once they were added to sediment. Moreover, it has been shown that ENPs can be very reactive and aggregate and agglomerate to form larger particles in seawater (i.e., (Cong, 2011)).

Nonetheless, Dabrin et al. (2012) also found that the ratio of C_{DGT} at different spiking levels was lower than the ratio of corresponding total cadmium concentrations in sediment. The insignificant effect of nominal concentration on DGT-bound Ag may be due to changes in the physical-chemical properties of sediment, such as pH. Increasing Cu and zinc concentrations in sediment caused the pH to decrease (Hutchins et al., 2007) due to (i) hydrolysis of spiked metal (ii) oxidative precipitation of iron in pore water and (iii) competition between protons H^+ and spiked metal for sorption sites on particulate phase (i.e., organic matter, AVS). Since sediment is a complex environment with presences of various

ligands, changes in pH may not directly result into releasing of metal ions. So, further study is required on how different parameters of sediment, such as pH, metal concentration, active sulfide, and organic matter affect C_{DGT} measurements of ENPs.

4. Conclusions

Few studies have addressed toxicity and bioaccumulation of metal nanoparticles in deposit feeders, especially comparing metal forms, nanoparticles size and shape. In this thesis, these factors were evaluated in two species from different taxa.

Nanoparticles were bioavailable to deposit feeders through sediment exposure. Based on DGT-measured Ag concentrations, released Ag ions from Ag NPs made a limited contribution to bioavailability of Ag NPs in marine sediment. It is a relatively new technique applied in sediment, and relevant theories are still under development. Dissolution of ENPs should be studied more in sediment since this is one of the key issues needed to differentiate ENPs from corresponding metal ions for hazard identification and exposure evaluation.

All tested nanoparticles were accumulated in *C. teleta* and *M. balthica* though with different ranking of Ag NPs and CuO NPs. However, the two species had different responses to sediment exposures of metal in different forms and nanoparticles with different sizes and/or shapes. In *C. teleta*, no differences among metal form or nanoparticles with different sizes were found in body burdens of Ag and Cu. The bioaccumulation of CuO NPs had comparable uptake rates and depuration rates among different Cu forms, CuO NPs' sizes. The nanoparticle shape mattered to the uptake and depuration kinetics of Cu in *C. teleta*. In soft tissue of *M. balthica*, bioaccumulation of Ag and Cu were both form- and particle size- dependent. Aqueous Cu ions were taken up more than CuO nanoparticles followed by micron-sized particles during 35 days of exposure. But no depuration was observed in accumulated Cu from CuO NPs or micron-sized particles (i.e., insignificant linear regression of body burden over time). In this thesis, different findings of uptake and depuration kinetics may reflect different binding sites of internalized nanoparticles; however, this will need to be investigated in further studies.

In this thesis, no toxicity of Ag or Cu was detected in *M. balthica* after exposures to metal in different forms or nanoparticles with different sizes or shapes. Similar mortality of *C. teleta* was only found after exposures to Cu in all forms, CuO NPs with different particles sizes and shapes (approximately 23% for all treatments in Paper III). This result suggests that toxicity of ENPs was only related to metal but not form, size or shape. This same hazard of ENPs as metal ions would facilitate the risk assessment of ENPs. In addition, delayed mortality of Cu (approximately 23%) may relate to its body burden in *C. teleta*, which should be investigated in further studies.

As demonstrated, characterization of ENPs is important for toxicological studies. But there is substantial uncertainty in states of metal nanoparticles in sediment after spiking because many factors may affect their properties (i.e., particle distribution and surface charge) and change the potential toxicity of ENPs to deposit feeders.

5. Acknowledgements

First and foremost I offer my sincerest gratitude to my supervisors, Gary T. Banta, Henriette Selck and Valery E. Forbes who have supported me during my PhD studies with their inspiration, patience and knowledge whilst allowing me the room to work in my own way. They helped me so much throughout my thesis with their encouragement and efforts, without them this thesis, would not have been completed or written. One simply could not wish for better or friendlier supervisors like them.

During my whole PhD research, I have had help from many people and without them I would never have been able to finish it. Anne-Grete Winding drive to collect water, sediment and animals which is a really fun trips and she always bought me what I needed for my studies. Michael Rabe encouraged me to handle AAS myself and never blamed me when I broke the machine. Anne Busk Faarborg, Rikke Guttesen, Anja Maria Holden Damsholt, and Klara Jensen provide their assistance during my studies. In addition, the Ecotox Lab of Anders Baun contributes a lot to my scientific experience.

Last but not least, it was excellent cooperation with Kristian Syberg who I learned skills and scientific thought from during our experiments. High appreciation belongs to Ecotox Group with Amalie Thit Jensen, Tina Ramskov, Annemette Palmqvist, Signe Pedersen, Linus Malmaquist, Cong Yi, Chengpang Pang, Mattia Meli, Jürgen Schmidt and Agnieszka Hunka.

I will also thank my family for their love.

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Paper I

Importance of characterizing nanoparticles before conducting toxicity tests

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Intgr. Environ. Assess. Manag. 2011. 7 (3), 502-503

I have ocntributed to this paper by:
Taking part in the development of the idea
Contributing to writing the manuscirpt

Published in *Integrated Environmental Assessment and Management* 7:3 (July 2011), pp. 502–503; doi: 10.1002/ieam.204

Published online June 20, 2011.

Importance of Characterizing Nanoparticles before Conducting Toxicity Tests

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Rapidly expanding growth in the field of nanotechnology has led to the development of numerous applications of nanomaterials in industrial (e.g., paints, electronics) and consumer (e.g., cosmetics, clothing treatments) products. These engineered nanoparticle (NP)-containing products have, however, the potential to release particles (single or aggregates) or ions by means of wastewater discharge into the aquatic environment. SCENIHR (2006) emphasized that the behavior of NPs is critically dependent on several particle characteristics, including size, surface area and surface reactivity, and that risk assessments for both human health and the environment have to be based on these characteristics. However, in practice, risks of NPs are in most cases assessed on the basis of their chemical composition alone and, to date, no widely accepted or well-defined risk assessment methods or test strategies exist explicitly designed for NPs.

There is a growing consensus on the necessity of proper and accurate characterization of NPs in environmental media and biological systems to ensure reliable and reproducible toxicity tests are performed. Without such characterization, nanotoxicity experiments will have limited value due to unknown variability in experimental conditions of the NPs (Warheit 2008). Some of the current divergent or conflicting results from nanotoxicological tests could also be better explained if there had been adequate characterization in all studies. However, exhaustive characterization of NPs is undoubtedly costly and time-consuming, and therefore, a sufficient but practical approach is needed. Some principal characteristics of NPs which have been considered to deserve quantification before conducting toxicity tests are size, shape, state of dispersion, physical and chemical properties (e.g., electronic and optical properties, chemical composition and reactivity), surface area, and surface chemistry (Powers et al. 2006). Whereas a significant number of papers list some of these characteristics for the powder or the initial dispersion media (usually in distilled water) few, if any, studies of aquatic nanotoxicity have provided a full characterization of the size distribution (especially hydrodynamic size), dispersion state (especially in biological media) or surface chemistry (like surface charge) of NPs in the actual test media. However, many NPs are likely to undergo significant size distribution or surface chemistry changes when they are transferred between media during experiments, such as from dispersion media (deionized water) to test media (e.g., sediment, freshwater, seawater, and cell culture media). Such changes may alter bioavailability or toxicity in ways that are not entirely understood.

We have characterized commercially available Ag NPs before conducting toxicity tests (Cong et al. unpublished data) and found a clear difference between the manufacturer's information (< 100nm and 2 to 3.5mm, respectively) and what we measured (20 to 200nm and 8 nm to 3mm in deionized water, respectively) for 2 Ag forms. This difference in size between that reported by the manufacturer and that measured in the laboratory was also observed by Scown et al. (2010). The reasons are most likely due to batch-to-batch variation during production, changes in material properties between synthesis and initial characterization, and particular experimental conditions when used (e.g., pH, ionic strength, and temperature). This observed variability highlights the importance of fully characterizing commercially obtained NPs before performing toxicity experiments, at the very least in the stock solutions used to prepare exposure treatments Given this sensitivity to experimental conditions, it is also important to characterize the NPs for each experiment conducted.

The preparation of stock NP suspensions for characterization before conducting laboratory experiments usually uses external mixing forces, like solvent dispersion, shaking, centrifugation, ultrafiltration, sonication, as well as surface modification and coatings to make NPs disperse evenly. All of these processes and treatments may change the properties of the NPs and may, therefore, be environmentally unrealistic compared with NPs released to the environment. Environmental factors, such as water pH, salinity and temperature, dissolved organic material, and natural competing cations, are likely to play important roles in determining the dispersion, toxic consequences, and compartment in which NPs are retained in the environment. Ideally, characterization of NPs should be performed under conditions as close as possible to the relevant exposure medium. For NPs intentionally or accidentally introduced into the aquatic environment, sediment is likely to act as a potential sink as it does for many chemicals. However, we know little about the state of sediment-associated NPs due to the limitation of techniques and methods for characterizing NPs in such complex and "dirty" media. We can start our exposures with nicely dispersed and well characterized NPs in a deionized water stock solution, but as soon as we add the solution to sediment we are working literally with a black box. Although we might expect NPs to become highly aggregated upon contact with sediment and therefore lose their tendency to behave differently than their chemically identical counterparts, our preliminary results suggest that this is not necessarily the case. Because the reactivity and toxicity of NPs are believed to be influenced by such features as their size, shape, surface coating, and other properties, we conclude that both the physical and chemical properties of NPs must be systematically and adequately defined before toxicological studies and risk assessment. The publication of toxicity test results should require that a characterization be performed in stock solutions used for testing. At the same time, there is a pressing need for the development of better methods for effectively characterizing NPs in complex environmental media (e.g., seawater, sediment) and living tissue.

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Paper II

Bioaccumulation and Effects of Silver amended to Sediment in forms of ions, nanoparticles and micron particles to a Marine Deposit Feeder, *Capitella teleta*

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I have ocntributed to this paper by:
Being the principle developer of the idea and design of the experiment
Being the principle investigator during the experimental work
Writing the manuscirpt

Bioaccumulation and effects of silver added to sediment as ions, nanoparticles and micron particles to a marine deposit feeder, *Capitella teleta*

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Abstract

The effects and bioavailability of sediment-associated silver were tested on *Capitella teleta* exposed to sediment amended with ionic Ag, 20 nm nanoparticles (AgNPs), 40 nm AgNPs, 100 nm AgNPs or micron-sized Ag particles at 3 silver concentrations for 14 days. Mortality was less than 10% for worms in all Ag treatments. No detrimental effects of any of the tested Ag forms were detected on growth rate or weight-specific feeding rate of worms at nominal exposure concentrations up to 100 µg/g dry weight sediment. Ag bioaccumulation in *C. teleta* increased with exposure concentrations but was independent of Ag form and particle size. After 14 days, approximately 3.6% of the sediment volume was processed by worms and packaged into fecal pellets. Fecal pellets contained more than 40% of the total added Ag, indicating a selective feeding behavior and retention of sediment-associated Ag (i.e., minimal loss to overlying water). A net transfer of a large amount of Ag into robust fecal pellets produced by *C. teleta* will likely change the fate of AgNPs in the aquatic environment.

Key words: Polychaete, sediment exposure, particle size dependent, feeding rate, re-distribution

INTRODUCTION

Engineered nanoparticles (ENPs) have received much attention during recent decades from both industry and environmental risk assessors. One of the remarkable properties of ENPs is their small particle size. As particle size decreases to less than 100 nm, novel physical-chemical properties are created for nanoparticles compared to larger particles containing the same metal core. For instance, there is an increased reactivity of NPs with small particle size because of the increased surface to volume ratio and an increase in particle-quantum effects (Baun et al., 2008). ENPs are produced with various particle sizes, particles shapes and surface properties to meet different technological uses, e.g., in drug delivery and in food packaging (Wijnhoven et al., 2009). It has been estimated that ENPs will be a trillion dollar market by 2015 (Maynard, 2006, Roco, 2004). Silver nanoparticles (AgNPs) have been used in water purification and surface coating of medicines for a long time due to their anti-bacterial property (Marambio-Jones and Hoek, 2010). Louma (2008) estimated that almost one-third of the AgNPs products on the market can release Ag or AgNPs which subsequently can enter the aquatic environment via wastewater discharge (Dowling, 2004, Benn and Westerhoff, 2008). Sediments are believed to be the final sink for AgNPs in the aquatic environment with an input of 195 – 1203 ng per kg sediment per year (Gottschalk et al., 2009). Thus, sediment dwelling organisms may be at high risk for exposure to and possible toxic effects of AgNPs.

AgNPs toxicity has been demonstrated for several species and includes effects such as phenotypic abnormalities and physiological dysfunction of aquatic vertebrate embryos during development (Wu et al., 2010, Choi et al., 2010). AgNPs have been shown to be bioavailable to and bioaccumulate in *Daphnia magna* from both water and dietary uptake routes, and the assimilation efficiency of AgNPs was higher than that of Ag ions through dietary uptake (Zhao and Wang, 2010). To our knowledge, however, few deposit feeders have been used for ecotoxicology studies of ENPs. Cong et al. (2011) found that Ag added to sediment as NPs (nominal < 100 nm) was more genotoxic and cytotoxic than ionic Ag in *Nereis (Hediste) diversicolor* exposed to 25 µg Ag/g dry weight sediment. Pang et al. (2012) found that mortality was twice as high in snails

exposed to sediment amended with 6 nm CuO NPs compared to snails exposed to sediment amended with ionic Cu. Buffet et al. (2011) found, however, no significant difference in accumulated Cu in *Scrobicularia plana* or *N. diversicolor* exposed to sediment spiked with Cu in different forms (i.e., ionic Cu and CuO NPs). Although ZnO NPs were found to impair feeding rate, egestion rate and assimilation efficiency of food in a freshwater snail, it is still not clear whether toxicity of ZnO was due to the metal or to the fact that it was in nanoparticle form (Croteau et al., 2011). The literature concerning deposit feeders and toxicity and bioaccumulation of NPs is still limited, and there is a pressing need for studies of this kind.

Capitella teleta (formerly known as *Capitella* sp. I) is an opportunistic species inhabiting organically enriched sediments near sewage outfalls, fish farms and other metal-polluted locations (Blake et al., 2009, Grassle and Grassle, 1974, Spies et al., 1989,Tsutsumi, 1990). *C. teleta* meets its nutritional requirements from the organic fraction of ingested sediment (i.e., attached bacteria or surface-bound organic coatings) (Lopez and Levinton, 1987) and it can process several times its body weight of sediment per day (up to 12 times) (Méndez et al., 2001). Populations of *C. teleta* can reach up to 400,000 individuals per m^2 in organically enriched sediment (Méndez et al., 1997). Organically enriched sediments are usually also sites of accumulation for hydrophobic compounds and metals. Hence, *C. teleta* may be at high risk of being exposed to sediment-associated Ag and AgNPs. In addition, it is well known that deposit-feeding organisms can affect the fate of sediment-associated contaminants through bioturbation (Hansen et al., 1999).

The aim of the present study was to investigate the hypothesis that toxicity and bioaccumulation of sediment-associated Ag to *C. teleta* is both form and particle size dependent. This was tested by exposing *C. teleta* to sediments amended with different Ag forms and AgNPs with different particle sizes for 14 days. Fate of Ag was analyzed by quantifying Ag in overlying water, sediment and fecal pellets.

METHODS

Sediment

Sediments were collected from Isefjorden (55°40'N, 11°47'E, Munkholm, Denmark)

on September 2010 after which they were sieved through 125 µm mesh in de-ionized water. Sieved sediments (≤125 µm) were thoroughly washed with seawater (31‰) 3 times before use. The water content of the sediment after this pre-treatment was 41.8% (± 0.4%; *n* = 5) determined by drying for 12 h at 105°C. The organic matter content was 2.8% (±0.2%, n = 5) determined by loss on ignition for 6 h at 550°C.

Test organism

The *Capitella teleta* used in this study were derived from laboratory cultures of worms that were originally collected in Setauket Harbor, New York, USA and identified as *Capitella* sp. I by J. Grassle (pers. com.). Worms were cultured in sieved, field-collected sediment (2 to 5 cm layer, grain size ≤ 250 µm) and seawater (31‰) at 17°C in our laboratory.

Ag nanoparticles synthesis and characterization (JRC)

Silver nanoparticles (AgNPs) were prepared via a modified Tollens process, consisting in the chemical reduction of the complex cation $[Ag(NH_3)_2]^+$ by sugars (Kvítek et al., 2005). Briefly, NH_4OH was added to 100ml of 2mM aqueous $AgNO_3$ solution under vigorous stirring to form the ammonia sugar complex. Subsequently, 100ml aqueous solution of 0.01M D-(+)-maltose monohydrate was added to the mixture. The reduction reaction of the silver by the sugar was then initiated by adding NaOH solution to increase the pH to approximately 11. The experiments were performed at room temperature (approx. 25°C) in a 250ml flask protected from light. By changing the concentrations of NH_4OH and NaOH used in the reaction mixture it was possible to vary in a controlled manner the final size of particles produced. In particular, the production of the 40nm particles required 0.4ml of 4N NH_4OH and 5.5ml of 1M NaOH while 100nm particles required 0.5ml of 4N NH_4OH and 2ml of 0.1M NaOH. The synthesized AgNPs suspensions were characterized by dynamic light scattering in their particle size distributions.

Ag spiked sediment

Silver nitrate ($AgNO_3$, Sigma-Aldrich, Germany), AgNPs (20 nm, 40 nm, and 100 nm nanoparticles coated with maltose) and micro-size Ag (range: 2-3.5 µm, Sigma-Aldrich, German) were used. A detailed characterization of micron-Ag has

been reported in Cong (2011). Briefly, micron-Ag had a wide size distribution from 8 nm to 3 µm and zeta potential of -49.0 mV (Cong et al., 2011).

The 3 AgNPs dispersions were prepared at 0.1 mg/ml. A solution of aqueous Ag ions was prepared at 1 mg /ml in MilliQ water (18.2Ω, Millipore) and the Micron-Ag dispersion was prepared at 0.3 mg/ml MilliQ water. Known volumes of stock solution/dispersions were transferred into clean sediment (≤ 125 µm) to get nominal concentrations of 10, 50 and 100 µg/g dry weight sediment (dw sed) for exposure. All Ag treatments and the control (i.e., no addition of Ag in any form) were placed on a shaking table for 24 h. Subsequently, all sediments were stored in the dark at 4°C for up to 10 d before use.

Experimental setup

Fifteen g dw sediment from each treatment was transferred into each replicate (100 ml volume beaker with 40 mm diameter) yielding a sediment depth of approximately 15 mm after which 60 ml of seawater (31‰) was added. Exposure systems were aerated for 24 h before exposure. One day prior to the start of exposure, worms were taken out of the lab cultures and allowed to defecate in clean seawater overnight. Eighteen worms were grouped randomly and were added to each of 4 replicates of a treatment (i.e., a total of 72 worms per treatment). Due to the large number of experimental systems to handle, replicates were started in staggered intervals with one replicate from each treatment set up every 3 days. All 64 replicate beakers were thus initiated over a 12 d period. All replicates were covered with parafilm to reduce evaporation and an air pump was provided for aeration. All replicates were placed at 17°C for 14 d with a light:dark cycle of 12 h:12 h.

After 14 d, worms were sieved out of the sediment (mesh size: 0.300 mm) using clean seawater (31‰) after which they were allowed to empty their guts for 12 h in clean seawater at 17°C in the dark. Sediment particles and fecal pellets from the exposure beakers were separated by differential settling (i.e., gentle swirling of the mixture in seawater) and checked under a dissecting microscope (Dai et al., 2012). All samples were stored frozen at -4°C until further analysis.

AAS measurements of Ag

The AAS methods used for Ag measurements followed Cong (2011). Briefly, samples for Ag measurements were lyophilized (Christ Alpha 1-2, Osterode, German) at -50°C overnight. Known amounts of samples (ca. 0.3 g dw sed, 0.3 g dw fecal pellets, 5 ml overlying water and 18 worms pooled of ca. 7.1 mg) were transferred into acid washed Weflon tubes and digested with 65% HNO_3 in a microwave oven (Milestone MLS-1200 Mega, Leutenkirch, Germany) by 250 W, 400 W, 650 W and 250 W for 6 min, respectively. Digested solutions were neutralized with NH_4OH and were subsequently measured on a graphite furnace atomic absorption spectrometer (GFAAS, GTA120, Varian, Australia) or flame atomic absorption spectrometer (FAAS, SpectrAA-220, Varian, Australia).

Endpoints

In the present study we measured mortality, volume-specific growth rate (SGR), weight-specific body burden (WSBB), weight-specific feeding rate (WSFR), Ag concentrations in fecal pellets, overlying water and sediment ($[Ag]_{FP}$, $[Ag]_{water}$ and $[Ag]_{sed}$).

The volume specific growth rate (SGR, % d^{-1}) was measured as the change of body volume (BV) of *C. teleta* over time (d, i.e., 14 d) according to (Forbes and Lopez, 1987)-SGR (%) $= \frac{\ln(BV14)-\ln(BV0)}{d} \times 100$. Body volumes were videotaped with a video camera mounted on a dissecting microscope. Body length (L) and surface area (A) were measured using an image-analysis program (SigmaScan Pro software, ver. 5.0.0., SPSS®). Assuming that worms are cylindrical in shape BVs were estimated as $BV = \pi A^2/4L$ (Self and Jumars, 1978). Worms were videotaped both at the beginning (BV_0) and termination of the exposure period (BV_{14}).

Weight-specific feeding rate (WSFR) was measured by the amount of fecal pellets divided by total worm biomass in each replicates and duration of 14 days.

Weight-specific body burden (WSBB) was measured on AAS by pooling all living worms together after 14 days.

Statistics

The choice of analysis of variance (ANOVA) or Kruskal-Wallis tests to analyze

$[Ag]_{FP}$ was based on a check of the assumption of homogeneous and normally distributed error terms. Analysis of co-variance (ANCOVA) corrected for BV_0 was used to detect effects of Ag form and nominal concentration on SGR, WSBB and WSFR. Student's *t*-test was used to compare measured endpoints between Ag exposure with pooled data (i.e., across both Ag forms and nominal concentrations) and control. In addition, due to the unbalanced experiment setup (i.e., missing data from 20 nm at the nominal concentration of 100 µg/g dw sed), the data selection for each statistical analysis is explicitly stated. SYSTAT 13.0 software (Chicago, USA) was used for all statistical analyses. The statistics methods are summarized in **Table 1**. A significance level of $p \leq 0.05$ was used throughout, and results are defined as marginally significant if $0.05 < p < 0.10$.

RESULTS

Characterization of AgNPs

The presence of AgNPs was observed in prepared suspensions (**Fig. 1**). The average hydrodynamic diameters of the three nanoparticles, characterized by DLS and expressed as intensity, were 21.2 nm, 44.3 nm and 97.4 nm for 20 nm, 40 nm and 100 nm, respectively.

Initial measured concentrations of sediment-associated Ag

The initial concentrations of sediment-associated Ag in the different treatments are presented in **Table 2**. There were no significant differences among Ag forms in initial measured concentrations of Ag at low (10 µg/g dw sed: p=0.141, Kruskal-Wallis), medium (50 µg/g dw sed: p = 0.509, ANOVA) or high (100 µg/g dw sed: p = 0.352, ANOVA excl. 20 nm treatment) nominal concentration levels, respectively. At high nominal concentration, the measured concentration of 20 nm was significantly lower (approximately 50 µg/g dw sed) than the rest of the treatments (Tukey test, $p< 0.001$). Thus, the treatment of 20 nm at the high nominal concentration was eliminated from exposure of *Capitella teleta*. The measured concentration of Ag in control sediment was below the detection limit of 10 ng/g dw sed.

Effects of Ag exposure

Mortality. Mortality was less than 10% in all Ag treatments (**Table S1**). All worms in

the control group survived after exposure, and the highest mortality was found in the treatment of aqueous ions at medium nominal concentration after 14 d (8.4% ± 7.1%). Without the 20 nm treatment and the control, no interactions of Ag form and nominal concentration was detected on arcsine transformed mortality data (Two-way ANOVA, $p = 0.960$). A reduced model was run without the interaction term. No effects of Ag form or nominal concentration on mortality were detected (One-way ANOVA, $p = 0.163$ for Ag form and $p = 0.794$ for nominal concentration).

Specific growth rate (SGR). Worms had positive growth rates in all treatments during exposure (**Fig. 2**). However, BV_0 were significantly different among replicates at day 0 of exposure (One-way ANOVA, $p = 0.013$). The initial body volumes were: 0.975 mm^3 (±0.093) in replicate 1, 1.198 mm^3 (±0.084) in replicate 2, 0.872 mm^3 (±0.067) in replicate 3 and 0.689 mm^3 (±0.082) in replicate 4 (n=270 per replicate). Thus analysis of SGR was corrected for BV_0. A significant linear relationship was detected between BV_0 and SGR (Linear regression, $p < 0.001$, Fig. S1) such that $SGR = -8.315 \times BV0 + 12.494$. The smaller the worms the faster the SGR during exposure, and the SGRs (% d^{-1}) were 3.4±1.8, 2.9±1.0, 5.1±1.2 and 7.2±1.5 in replicate 1, 2, 3, and 4 after 14 d, respectively.

Without 20 nm treatments and control, no interaction of Ag form and exposure concentration was detected on SGR after 14 d (Blocked two-way ANCOVA, $p = 0.328$). A reduced model was run without the interaction term. No effects of Ag form or nominal concentration were detected on SGR (Blocked one-way ANCOVA, $p = 0.334$ for Ag form and $p = 0.296$ for nominal concentration). In addition, exposure to Ag had no significant effect on SGR (Student's *t*-test, $p = 0.854$). The average SGR of worms was 4.6 % d^{-1} (± 2.2 %, n = 1031) by pooling data from all treatments.

Weight-specific feeding rate. The weight-specific feeding rate (WSFR) was similar in all treatments (**Fig. 3**). Excluding the 20 nm and control, no interactions of Ag form and nominal concentration were detected on WSFR after 14 days of exposure (Blocked two-way ANCOVA, $p = 0.966$). A reduced model was run without the interaction term. No significant effects of Ag form or nominal concentration were detected on WSFR (Blocked one-way ANCOVA, $p = 0.747$ for Ag form and $p = 0.177$

for nominal concentration). In addition, Ag exposure did not have an effect of WSFR on worms after 14 d (Student's t-test, p = 0.864). The WSFR was 6.4±1.7 mg dw fecal pellets/mg dw worm/d (n = 60) by pooling data from all treatments.

Weight-specific body burden. Ag was detected in worms from all treatments except those from the control group in which Ag was below the detection limit of 0.8 µg Ag/g dw worm (**Fig. 4**). Excluding the 20 nm treatments, no interactions of Ag form and nominal concentration were detected on WSBB (Blocked two-way ANCOVA, p = 0.983). A reduced model run without the interaction term showed no significant effect of Ag form on WSBB after 14 d exposure (Blocked one-way ANOVA, p =0.977). However the WSBB increased significantly with increasing exposure concentrations as expected (Blocked ANCOVA, p = 0.007). The average WSBBs increased 2.8 times at medium nominal concentration and 4.1 times at high nominal concentration when compared to WSBB at low nominal concentration.

Distribution of Ag in water, sediment, and fecal pellets on day 14

Ag concentration in overlying water. No Ag was detected in overlying water after 14 days of exposure. The detection limit of Ag was 6.0 µg/L.

Ag concentrations in fecal pellets. Excluding 20 nm treatments and the control, no significant interaction of Ag form and nominal concentration was detected on $[Ag]_{FP}$ after 14 d exposure (**Fig. 5**, Two-way ANOVA, p = 0.102). A reduced model was run without an interaction term. There was a significant effect of nominal concentration but not of Ag form on $[Ag]_{FP}$ (One-way ANOVA, $p < 0.001$ for nominal concentration and p = 0.130 for Ag form, respectively). The concentration of Ag in fecal pellets increased with increasing nominal Ag concentration: $[Ag]_{FP}$ increased 5.1 times at medium nominal concentration (635.2±65.1 µg Ag/g dw fecal pellets) and 10.7 times at high nominal concentration (1323.2±96.7 µg Ag/g dw fecal pellets) when compared to $[Ag]_{FP}$ at low nominal concentration (123.4±16.6 µg Ag/g dw fecal pellets) (Fig 4).

Ag concentration in sediment. The $[Ag]_{sed}$ increased with increasing nominal Ag concentrations in sediment regardless of Ag form (Fig. 6). Without 20 nm and control treatments, no significant interaction of Ag form and nominal concentration was detected on $[Ag]_{sed}$ on day 14 (Two-way ANOVA, p = 0.476). A reduced model was

run without an interaction term. A significant effect of nominal concentration was detected on $[Ag]_{sed}$ but not Ag form (One-way ANOVA, $p < 0.001$ for nominal concentration and $p = 0.279$ for Ag form). Compared to measured Ag concentration at day 0, $[Ag]_{sed}$ decreased approximately 30.5% after 14 days exposure, to give 6.2±0.9 µg Ag/g dw sed, 28.8±4.3 µg Ag/g dw sed and 56.8±14.0 µg Ag/g dw sed at low, medium and high nominal concentration, respectively.

Ag Budget. After 14 days of exposure of *C. teleta*, Ag was mainly distributed in sediment and fecal pellets (Table 3). There was approximately 52.1% Ag left in sediment and approximately 42.1% Ag was transferred into fecal pellets. The amount of accumulated Ag in worm tissue was less than 0.01% of the total Ag added at the start of the experiment. Approximately 5.8% Ag was lost during sample preparation.

DISCUSSION

In the present study, no toxicity of Ag in all forms was found (less than 10% mortality) in *C. teleta* exposed for 14 days. Based on the reported environmental concentrations of Ag in sediment samples obtained downstream of wastewater treatment plants receiving industrial sources so far (11 - 24 µg Ag/g dw sed) (Lytle, 1984), neither AgNPs nor Ag can cause lethal effects on *C. teleta* at highly polluted sites in environment.

Ag was documented to be one of the most toxic metals to invertebrates in marine and estuarine environments (Bryan, 1984). AgNPs and released Ag ions may interrupt normal metabolic activities, disrupt membrane permeability, and even cause DNA damage in exposed organisms (Kim et al., 2012, Cong et al., 2011, AshaRani et al., 2008, Sondi and Salopek-Sondi, 2004). DNA damage resulting from exposure to Ag in different forms suggests that the same mode of action may be involved for both aqueous Ag ions and AgNPs (Cong et al., 2011). However, toxicity of Ag is affected by several factors including species, food availability, exposure scenario and test media (i.e., freshwater vs seawater). In a study of toxicity of dissolved Ag to polychaete, *Pomatoceros triqueter*, no significant mortality was found up to 400 µg/L during 49-d water exposure (Vovelle and Grasset, 1991). After a 48 h exposure in a static renewal system with filtered hard fresh water and food, An LC_{50} of 8 µg/L is

reported for the daphnids, *Daphnia pulex*; while an LC_{50} of 160 µg/L is reported for the daphnids, *Ceriodaphnia dubia* (Griffitt et al., 2008). For *C. dubia*, LC_{50} of Ag decreases further to 0.5 µg/L after a 48-h exposure in a moderate hard water (Bielmyer et al., 2002). The toxicity of both Ag NPs and Ag ions is made difficult in the presence of common ligands such as Cl^-, S^{2-} and PO_4^{3-} in test media, given these ligands can decrease the concentration of Ag ions and thus the toxicity (Xiu et al., 2011). Thus, the nominal concentration of 100 µg/g dw sed too low to cause mortality of *C. teleta* due to the ligands in marine sediment, the test species or both in the present study.

To our knowledge, the present study was the first published result on toxicity of sediment associated Ag on *C. teleta*. This species has showed some tolerance to other metals. No mortality of *C. teleta* was found in exposure to extremely polluted sediment with Cd (up to 534 µg/g sed), Ni (up to 763 µg/g sed) and Pb (870 µg/g sed) (Horng et al., 2009). The apparent tolerance of *C. teleta* to Ag may relate to effective detoxification pathways in the species based on published results on other metals. For instance, in a study of intracellular distribution of accumulated metals in *C. teleta*, Goto and Wallace (2007) reported that less than 6% of accumulated Cd and Zn bound in metal sensitive fractions (i.e., with heat denaturable proteins) and up to 65% of accumulated Cd and Zn was stored as detoxified forms (i.e., with heat-stable proteins) after a water only exposure to 50 µg Cd/L and 86 µg Zn/L. Induction of metallothionein and -like proteins were significantly found in *C. teleta* after a water only exposure to Cu up to 200 µg/L (Suriya et al., 2012). In addition, excretion of accumulated Cd was found in *C. teleta* that approximately only 30% Cd was left in worms after 6 days (Selck et al., 1998). This was an active excretion of *C. teleta* ($t_{½}$ = 3 day) based on a common depuration rate of 1-5% corresponding to a weight-specific half-life of 14 – 69 days (Luoma and Rainbow, 2008). Thus, *C. teleta* may detoxify accumulated Ag through intracellular redistribution from critical sites to detoxified forms, binding with metallothionein and –like proteins and active excretion. García-Alonso et al. (2011) has been reported in *N. diversicolor* that Ag accumulated from Ag ions was mainly associated with metallothionein and –like proteins; while Ag

accumulated from AgNPs was mainly with metal organelles, which mean different detoxification pathways were involved. Hence, detoxification mechanisms of aqueous Ag ions and AgNPs should be studied further in *C. teleta* to understand its seemingly high tolerance to Ag in different forms.

In the present study, neither Ag form nor nominal concentration was found to affect SGR or feeding rate. The weight-specific feeding rate in the present study (6.4 mg dw FP/mg dw worm/d) was close to a reported range of 8 – 10 mg sed/mg worm/d observed by Forbes (1984). Generally, aquatic organisms may change their feeding behaviors to support the nutrient requirements for growth under stress. In a study of rainbow trout exposed to Ag spiked food, growth was not affected but feeding rate increased significantly after 24-, 43- and 58-days exposure at 3000 µg/g ww food (Galvez and Wood, 1999). By inhibiting digestive enzyme secretion in digestive gland cells, Ag may retard growth rate of the cuttlefish (*Sepia officinalis* L.) as a consequence of decreased assimilation efficiency of nutrients (Le Bihan et al., 2004). However, inhibition of enzyme secretion does not seem to be a factor in *C. teleta* exposed to Ag since neither SGR nor WSFR were affected by Ag. No effects of Ag exposures were detected on growth and feeding rate of *Ceriodaphnia* spp. and *Simocephalus* spp. (exposure up to 540 ng Ag /L) (Hook and Fisher, 2009). It suggested that either the high nominal concentration of Ag may be not high enough to impair assimilation efficiency of food or other action of mode may exist in *C. teleta*.

There was a negative relationship between worm size and growth rate which was 4.6% d^{-1} starting with an initial body volume of 0.934 mm^3 in the present study. In a study of the lift traits of *C. teleta* by Ramskov and Forbes (2008), SGR varied from 0 to 50% d^{-1} for *C. teleta* starting with a volume of 0.005 mm^3, which related negatively with time and positively with organic matter content. Linton and Taghon (2000) found that the growth rate of *C. teleta* decreased from approximately 23% d^{-1} to less than 10% d^{-1} with a reduction of organic matter quantified as total carbon and total nitrogen concentrations (51.2 – 11.5 mg TC/g dw sed and 5.5 – 1.5 mg TN/g dw sed). The quality of sediment can be determined based on measures of organic carbon content, organic nitrogen content, amino acids, essential fatty acid content and the amount of

labile organic matter (Tenore, 1977a, Tenore, 1977b, Taghon, 1981, Cheng et al., 1993). The relatively low growth rate of *C. teleta* (4.7% d^{-1}, 2.8% organic matter content) may due to low organic matter quality in the present study. However, we did not determine the quality of sediment organic matter, and the relationship between sediment organic matter quality and growth rate needs to be further investigated.

In the present study, the weight-specific body burden of Ag increased with nominal concentration of Ag in sediment, which indicated that sediment associated Ag ions and Ag NPs was bioavailable to *C. teleta*. In general, this species can accumulate metals in sediment exposures through 3 uptake routes, (1) ingestion of sediment and assimilation of sediment associated metal in guts; (2) from pore water and (3) from overlying water. The latter two routes involve diffusions of free ions across worm body surface (Selck et al., 1998). Selck et al. (1998) reported that 95% body burden of Cd in *C. teleta* was accumulated from sediment ingestion of 25.7 µg Cd/g dw sed for 5 days. It was also reported that only 65 to 95% Ag was accumulated by ingesting Ag spiked sediment in a marine polychaete, *Nereis succinea* which exposed to approximately 40 µg Ag/g dw sed (Wang et al., 1999). *C. teleta* is a species living in tubes and water is exchanged with overlying water during irrigation in order to increase oxygen content (Aller, 1982, Cammen et al., 1987). However, tubes may prevent the intimate contact with Ag dissolved in pore water. Since no Ag was measureable in overlying water in the present study. Thus, we believed that the accumulation of aqueous Ag ions and AgNPs was mainly determined by sediment ingestion of C. *teleta*.

In *C. teleta*, the bioaccumulation of Ag did not depend on Ag forms in the present study. The same result has also been observed in the polychaete, *N. (H.) diversicolor*, that both Cu and Ag were accumulated at the same level after water only and sediment exposures to aqueous ions, ENPs and micron sized particles (Buffet et al., 2011, Cong et al., 2011, García-Alonso et al., 2011). However, form dependent bioaccumulation has been commonly observed in other species, such as *Peringia ulvae*, *Lymnaea stagnal* and bivalves (Khan et al., 2012, Canesi et al., 2011). Form independent bioaccumulation is not clear and will be interesting to investigate in

further study.

After a 14-days exposure, Ag was detected in fecal pellets, sediment and *C. teleta* tissue. As a selective feeder, *C. teleta* preferentially feeds on 2 – 40 μm sediment particles with high nutrient values (Lopez and Levinton, 1987, Madsen et al., 1997, Horng and Taghon, 1999). In the present study, a total of 3.6% sediment was processed and packed as fecal pellets in all treatments, which contained 42.1% Ag added at the experimental beginning. The Ag was concentrated in fecal pellets by 19.9, 22.1 and 23.3 times greater than sediment ($[Ag]_{FP}$:$[Ag]_{sed}$) at low, medium and high nominal concentrations respectively. We seldom observed the disaggregation of fecal pellets in the gut of *C. teleta*. Fecal pellets have a robust structure and their half-life range from 5 to 33 years in aquatic environment (Gallagher and Keay, 1998). The fecal pellets-Ag may not be significantly released into surrounding environment through an abiotic process because less than 5% loss of polycyclic aromatic hydrocarbons from fecal pellets of *C. teleta* (Horng and Taghon, 2001). On the other hand, it has been reported that fecal pellets can only be consumed by some deposit feeders (i.e., *Hydrobia* spp. and *Amphicteis scaphobranchia*) after aging and disaggregation (Newell, 1965, Levinton and Lopez, 1977, Taghon et al., 1984). *C. teleta* has been observed to re-feed both disaggregated and intact fecal pellets but the worm body shrinks significantly by approximately 10% d^{-1} (Phillips and Tenore, 1984). The positive growth of worms in the present study mean that fecal pellets were not re-fed. Therefore, fecal pellets-Ag is not bioavailable before disaggregation once Ag is transferred into fecal pellets.

C. teleta is an active sediment-dwelling deposit feeder that ingests several times its own weight in sediment per day (Lopez and Levinton, 1987). Here we found that *C. teleta* processed about 6 times its own weight per day. In combination with the high population size of the species up to 400,000 individual/m^2 (Tsutsumi, 1990, Méndez et al., 1997), *C. teleta* could transfer a high percentage of the sediment-associated Ag (both AgNPs and other Ag forms) into fecal pellets in the field. Thus, *C. teleta* populations play a significant role to the exposure scenario of other deposit feeders to sediment-associated Ag in aquatic environment.

CONCLUSIONS

The present study demonstrated that both Ag ions and Ag particles of all sizes were bioavailable and accumulated in the deposit feeder, *Capitella teleta*, and that accumulation was independent of Ag form added to the sediment. No effects of Ag in different forms were observed in *C. teleta* after exposure for 14 days to concentrations up to about 90 µg/g sed. After 14 days, worms packed more than 40% of the total amount of Ag added to the sediment into fecal pellets indicating that this species may play an important role in the removal of Ag from the sediment. The presence of *C. teleta* with high population size and high feeding rate will, however, change the fate of AgNPs by repackaging the Ag into robust fecal pellets. However, uncertainties remain with regard to the contribution of released Ag ions both to the bioavailable fraction of Ag NPs in sediment and with regard to the uptake of AgNPs in the gut of *C. teleta*. More studies should focus on and experimentally manipulate the released metal ions from ENPs both in environmentally relevant compartments, such as sediments, and in the gut of exposed organisms.

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Acknowledgements

This work was supported by Roskilde University (RUC) and is done in cooperation

with the EU NanoReTox project (7th Framework Programme, Grant Agreement no. CP-FP214478-2). The authors gratefully acknowledge to technicians working at the Ecotoxicology Lab for their technical guidance and assistance during experiments.

FIGURE LEGENDS

Fig. 1. Characterization of AgNPs by dynamic light scattering, images of 20 nm (a), 40nm (b), 100 nm (c) and hydrodynamic size distribution of three AgNPs expressed by intensity (d).

Fig. 2. Specific growth rate (SGR, % d^{-1}) of *Capitella teleta* exposed to sediment-associated Ag in various forms after 14 d (n = 4 with 18 ind. per replicate). Error bars represent ± 1 standard deviation.

Fig 3. Weight-specific feeding rate (mg dw fecal pellets/mg dw worm/day) of *Capitella teleta* exposed to sediment-associated Ag in various forms after 14 ds (n = 4). It was estimated as the total sediment processed divided by the population biomass in each beaker divided by 14 d. Error bars represent ± 1 standard deviation.

Fig. 4. Weight specific body burden of Ag (µg/g dw worm) in various forms in *Capitella teleta* after exposure of 14 days (n=4). Error bars represent ± 1 standard deviation.

Fig. 5. The Ag concentrations measured in fecal pellets of *Capitella teleta* on day 14 (µg/g dw FP, n = 4). Error bars represent ± 1 standard deviation.

Fig. 6. The Ag concentrations measured in sediment on day 14 (µg/g dw sed, n = 4). Error bars represent ± 1 standard deviation.

TABLES

Table 1. Summary of statistical methods, data selection and results on measured endpoints in the present study.

	Ag form × Conc.[a] (without 20 nm or control)	Ag form (without control)	Conc. (without control)	Ag exposure *vs*. control
Mortality	-[b] (Two-way ANOVA)	- (One-way ANOVA)	- (One-way ANOVA)	- (Student's t-test)
SGR	- (Blocked two-way ANOVA)	- (Blocked one-way ANOVA)	- (Blocked one-way ANOVA)	- (Student's t-test)
WSFR	- (Blocked two-way ANOVA)	- (Blocked one-way ANOVA)	- (Blocked one-way ANOVA)	- (Student's t-test)
SBB	- (Blocked two-way ANOVA)	- (Blocked one-way ANOVA)	**[c] (Blocked one-way ANOVA)	- (Student's t-test)
$[Ag]_{FP}$	- (Two-way ANOVA)	- (One-way ANOVA)	** (One-way ANOVA)	N.M [d]
$[Ag]_{sed}$	- (Two-way ANOVA)	- (One-way ANOVA)	** (One-way ANOVA)	N.M

a. Conc. refers to nominal concentration of Ag (μg/g dw sed).
b. "-" refers to no significant effects with $p > 0.100$.
c. "**" refers to significant effects with $p < 0.050$.
d. "N.M" refers to no statistics was run.

Table 2. Initial measured Ag concentrations in sediments of different treatments (n=3).

Conc.[a]	Aqueous ion	Nanoparticles			Micron
		20 nm	40 nm	100 nm	
10	9.9 ± 0.3	8.4 ± 0.5	8.7 ± 0.5	8.4 ± 1.4	8.9 ± 0.6
50	41.6 ± 1.9	40.0 ± 1.9	39.2 ± 2.2	41.0 ± 2.8	41.8 ± 0.9
100	88.5 ± 3.6	N/E[b]	83.3 ± 7.9	79.5 ± 2.1	84.3 ± 7.3
control	0.0 ± 0.0				

a. Conc. refers to nominal concentration of Ag (μg/g dw sed)
b. N/E: no exposure

Table 3. Silver budget and distribution of Ag in exposure treatment after 14 days (n=4).

Ag form	Nominal conc.[a]	Day 0	Distribution of Ag on day 14 (%)[b]		
		Total Ag [µg]	Sediment	Fecal pellets	Loss
Aqueous ions	10	226.4±12.1	51.0±5.9	34.9±9.6	14.2±8.6
20 nm	10	230.0±22.2	52.8±10.9	39.1±5.3	8.1±9.2
40 nm	10	200.5±13.2	54.1±5.3	27.6±3.0	18.4±7.8
100 nm	10	192.2±12.5	53.9±8.7	43.5±10.9	1.1±6.5
Micron	10	204.0±27.1	52.3±3.9	38.2±12.1	9.2±13.3
Aqueous ions	50	1038.1±183.8	56.5±6.4	42.3±16.0	1.2±19.5
20 nm	50	920.1±123.8	49.3±8.3	49.1±18.4	3.9±13.5
40 nm	50	901.3±49.1	55.7±4.0	43.0±3.3	1.3±5.4
100 nm	50	943.9±183.8	59.8±6.4	33.9±16.0	6.3±19.5
Micron	50	1054.7±270.7	51.4±6.7	45.2±20.6	3.4±25.7
Aqueous ions	100	2036.2±262.7	53.5±9.1	49.3±20.5	-2.8±12.9
20 nm	100	-[c]	-	-	-
40 nm	100	1915.2±137.8	51.7±2.5	39.7±8.6	8.6±7.2
100 nm	100	1827.8±217.3	49.9±15.0	45.5±18.6	7.0±11.9
Micron	100	1939.3±96.1	37.2±4.7	57.4±23.3	5.4±5.0

a. Nominal conc. refers to the nominal concentration of Ag (µg/g dw sed).
b. No Ag was detected in overlying water phase; and amount of Ag in worm tissue was less than 0.01%. The distribution of Ag in sediment and fecal pellets on day 14 was calculated by percentage of Ag mass in these two compartment divided by total Ag amount on day 0. Loss was expressed as difference in Ag budgets at beginning and termination of the exposure.
c. '-' refers to no exposure of worms to 20 nm at high nominal concentration.

FIGURES

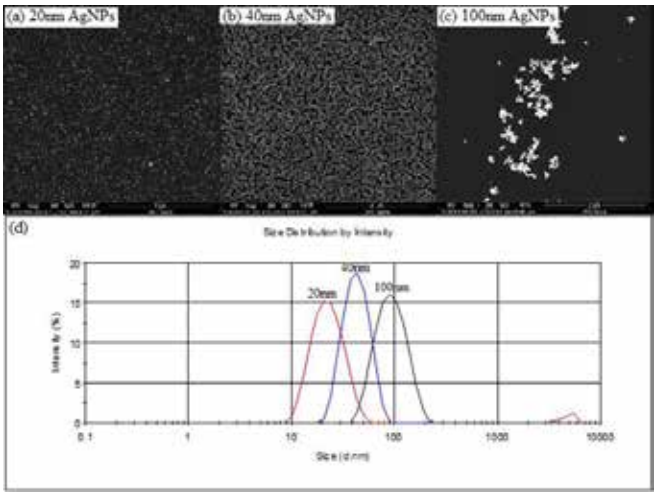

Fig. 1

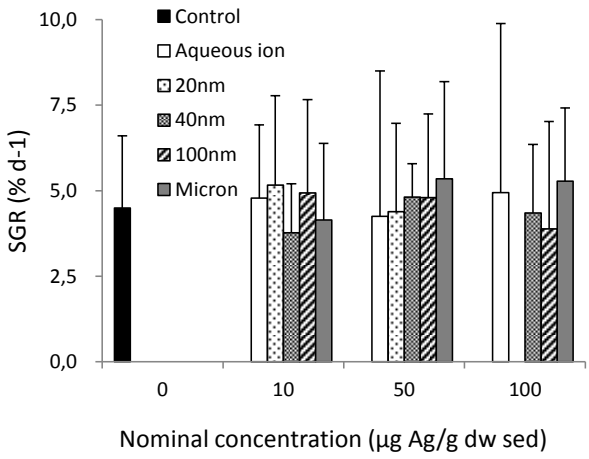

Fig. 2

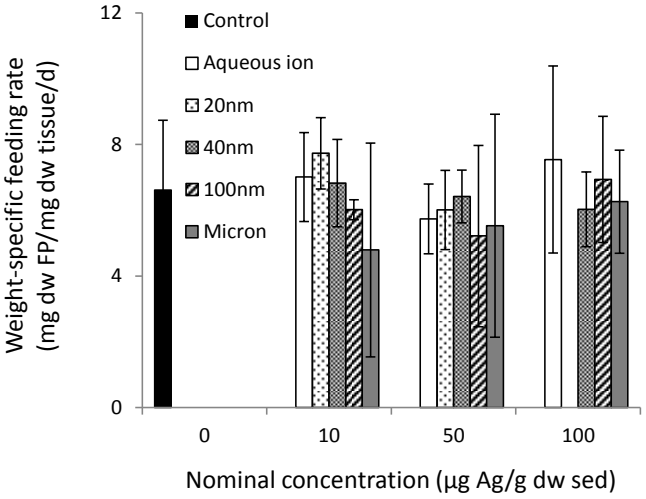

Fig. 3

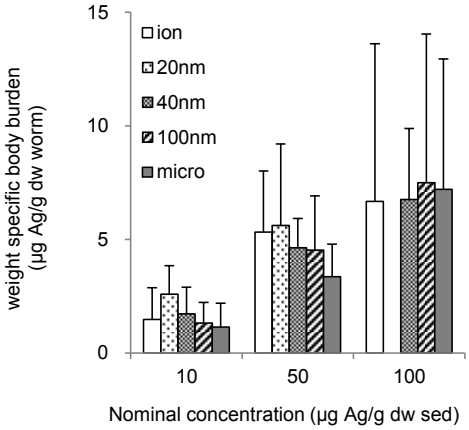

Fig. 4

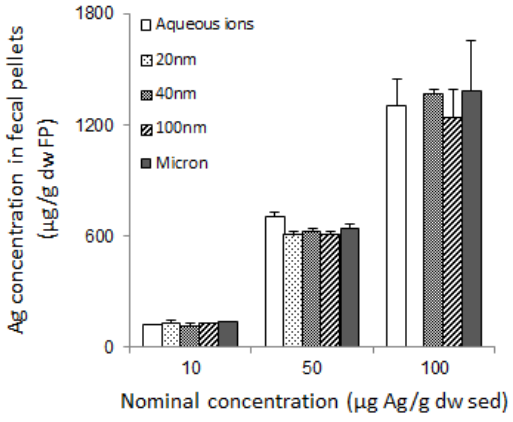

Fig. 5

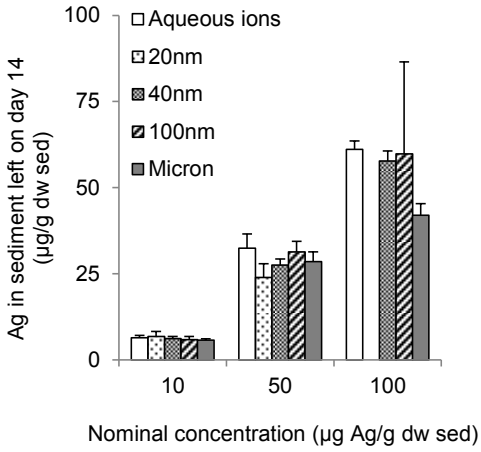
100
75
50
25
0
Aqueous ions
20nm
40nm
100nm
Micron
Ag in sediment left on day 14 (µg/g dw sed)
10
50
100
Nominal concentration (µg Ag/g dw sed)

Fig. 6

SUPPLYMENTAL DATA

Table S1. Mortality of *Capitella teleta* exposed to clean or Ag amended sediment after 14 d (n = 4).

Nominal.[a]	Aqueous ion	nanoparticles			Micron
		20 nm	40 nm	100 nm	
10	4.2 ± 5.3	1.4 ± 2.8	5.6 ± 7.9	4.3 ± 2.9	2.8 ± 5.6
50	8.4 ± 7.1	1.4 ± 2.8	0.0 ± 4.5	0.0 ± 0.0	4.2 ± 5.3
100	6.9 ± 8.3	N/E[b]	6.9 ± 8.3	2.9 ± 3.3	2.9 ± 3.3
control	0.0 ± 0.0				

a. Nominal. Refers to nominal concentration of Ag (µg/g dw sed)

b. N/E: no exposure

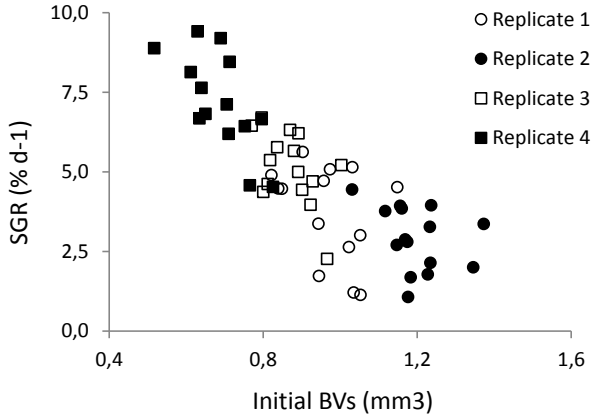

Figure S1. Specific growth rate (SGR, % d^{-1}) of *Capitella teleta* versus initial body volumes (BVs) of worms in different replicates.

Paper III

Toxicity and biokinetics of copper in various forms and copper oxide nanoparticles in different shapes in the deposit feeder, *Capitella teleta*

Lina Dai, Gary T Banta, Henriette Selck, Valery E Forbes

I have ocntributed to this paper by:
Being the principle developer of the idea and design of the experiment
Being the principle investigator during the experimental work
Writing the manuscirpt

Toxicity, uptake and depuration kinetics of copper in various forms and copper oxide nanoparticles in different shapes in the deposit feeder, *Capitella teleta*

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Abstract:

In the present study, the deposit feeder, *Capitella teleta*, was used to investigate the toxicity and effects of copper added to sediment in three forms: Aqueous ions, 100 nm copper oxide nanoparticles (CuO NPs) and micron-sized CuO particles; and CuO NPs in three shapes: spheres, spindles and rods. During both the exposure and depuration periods, low mortality of worms was observed and surviving worms showed positive growth. No significant differences in either endpoint as a function of Cu form or CuO NP shape was detected. Cu added in all treatments was bioavailable and accumulated in *C. teleta* during 7 d of exposure. The weight specific body burden (µg Cu/g dry wt worm) was 13.3 ± 5.5 for controls (no Cu added), 65.5 ± 30.6 for Aqueous ions, 55.0 ± 33.5 for 100 nm NPs, 135.3 ± 40.0 for micron-sized particles, 57.8 ± 16.4 for spheres, 143.0 ± 43.2 for spindles, and 235.4 ± 26.9 for rods after 7 d of exposure. Cu was completely depurated in all treatments after 7 d in clean sediment. No significant effects of Cu form or CuO NP shape were detected on depuration rate constants in worms. Positive net uptake rates in all treatments indicate that Cu added to sediment as CuO NPs has similar bioaccumulation potential as Cu added in aqueous form or as micron-sized particles for *C. teleta*. The interpretation of bioaccumulation patterns of NPs with different properties (size and shape) may change depending on how the dose is expressed.

Keywords: Polychaete, bioaccumulation, uptake, depuration, marine sediment

1. Introduction

With the rapid development of nanotechnology, metal-based engineered nanoparticles (ENPs) have been applied in numerous commercial products such as cosmetics, textiles and medicine. Engineered nanoparticles refer to particles that are smaller than 100 nm in at least one dimension that are produced for commercial purposes. It has been demonstrated that ENPs and corresponding metal ions released from ENPs, can enter the aquatic environment via sewage plant (Dowling, 2004, Benn and Westerhoff, 2008). As for many pollutants, sediment is believed to be an important repository for ENPs in aquatic environments. Thus, sediment-dwelling organisms may be at high risk for exposure and subsequent toxicity related to ENPs.

One of the remarkable properties of ENPs is their particle size. As particle size decreases to nano-scale, novel physical-chemical properties are created for nanoparticles compared to bulk particles with the same metal core. For instance, there is increasing reactivity of ENPs as their particle size decreases because of increased surface to volume ratio and increased particle-quantum effects (Nel et al., 2006). These novel properties may lead to unexpected biological effects in aquatic organisms. For instance, Cong et al. (2011) found that silver NPs were more genotoxic than ionic Ag in *Nereis diversicolor* exposed at 25 µg Ag/g dry wt sediment. Pang et al. (2012) found that mortality caused by exposure to 6 nm copper oxide nanoparticles (CuO NPs) was twice as high as a similar concentration of ionic Cu.

Recently, more attentions have been paid on bioaccumulation potential of metal-bearing NPs which may cause chronic toxicity in the field. In laboratory experiment, bioaccumulation of Ag NPs is found in *Daphnia magna* from both water and diet, and assimilation efficiency of Ag NPs was higher than that of Ag ions through dietary uptake (Zhao and Wang, 2010). Then, Khan et al. (2012) found that low body burden

of Ag NPs in *Peringia ulvae* and *Lymnaea stagnails* was due to low uptake but high elimination of Ag NPs than Ag ions. It suggests form or particle size dependent bioaccumulation may be resulted of different uptake kinetics in test organisms. However, no effects of metal form or particle size were found in bioaccumulation of metal in deposit feeders, *N. diversicolor* or *Scrobicularia plana* (Cong et al., 2011, Buffet et al., 2011). Little is known how these species uptake and eliminated metals in different form and metal-bearing particles.

In addition to particle size, another property of ENPs that may influence toxicity and bioaccumulation is particle shape. Engineered nanoparticles with different shapes have been developed for application to nanomedicine, sensing, catalysis, etc. Few studies have been conducted on effects of NPs shape on their toxicity and bioaccumulation in deposit feeders. The elongated TiO_2 (44 nm × 1500 nm) NPs cause approximately 10% mortality and spherical TiO2 (11 nm) NPs cause more than 50 % mortality of *Escherichia coli* (Simon-Deckers et al., 2009). Pal et al. (2007) found that truncated triangular Ag NPs can inhibit bacteria (*E. coli*) more effectively than Ag NP spheres or rods. At the same concentration, needle-shaped hydroxyapatite delayed embryo hatching ratio of zebrafish more than rod-shaped hydroxyapatite after 72 h (Zhao et al., 2012). Therefore, there is a pressing need to collect toxic data of NPs in different shape on deposit feeders.

Deposit feeders are organisms that meet their nutritional requirements by processing a large volume of sediments. Deposit feeders may be exposed to ENPs in two routes: through body surface uptake, transporting ENPs adsorbed on the body surface through cell membranes; and dietary uptake, transporting ENPs in ingested food (i.e., sediment) into gut epithelium cells. *Capitella teleta* (formerly *Capitella* sp. I) is an opportunistic species inhabiting organically enriched sediment near sewage outfalls,

fish farms and other organically polluted locations (Blake et al., 2009, Grassle and Grassle, 1974, Spies et al., 1989, Tsutsumi, 1990). *C. teleta* feeds selectively on fine-grained, organic-rich particles. Depending on the sediment organic matter content, population size can vary from a few to up to 400,000 individuals per m^2 in the field (Tsutsumi, 1990, Méndez et al., 1997). *C. teleta* is a well-studied species with respect to its survival, growth, feeding rate and other life-history traits (Grassle and Grassle, 1974, Ramskov and Forbes, 2008). It processes several times its body weight in sediment per day (up to 12 times), and therefore plays an important role in sediment biogeochemistry and sediment-associated contaminant turnover, particularly where it occurs in high densities (Méndez et al., 2001).

The aim of the present study was to investigate whether bioavailability and toxicity of sediment amended with copper (Cu) depended on the form in which Cu was added to sediment (i.e., as Cu ions, CuO nanoparticles or CuO micron-sized particles) and on nanoparticle shape (i.e., spheres, spindles, or rods). Toxicity was estimated as survival and growth. In order to better understand bioaccumulation, we quantified net uptake rates (k_u) and depuration rates (k_d) of these Cu forms in *C. teleta*.

2. METHOD

2.1 CuO NPs characterization

Both plasma synthesized 100 nm NPs (CuO nano-powder with nominal size of <100 nm, Intrinsiq Materials Ltd) and micron particles (CuO powder <5 µm, cat. #20, 884-1, assay 98%, Sigma–Aldrich, Denmark) were exactly same as Pang (2011). In brief, these two commercial particles were both poly-dispersed in prepared suspensions (1.3 mg particles/ml in MilliQ water) by transmission electron microscopy (Hitachi H-7100, 100 kV). The average hydrodynamic size was 204 nm and 813 nm characterized by Zetasiser Nano ZS for 100 nm and micron-sized CuO particles,

respectively. In addition, surface charge of 100 nm and micron-sized CuO particles were +42 mV and -20 mV, respectively. CuO NPs in different shapes (i.e., spheres, rods and spindles) were provided by Nature History Museum (UK). The dimension of spheres, rods and spindles were as 7 nm, 10×50 nm and 20×300×1000 nm, respectively in addition to positive surface charges of +43.4, +37.1 and +44.1 mV, respectively (Ramskov T, personal communication).

2.2 Sediment amended with Cu

Sediments were collected from Isefjorden (55°40'N, 11°47'E, Munkholm, Denmark) on September 2011 and subsequently sieved in distilled water to kill associated meiofauna. Sieved sediments (≤ 125 µm) were washed twice with seawater (31‰ salinity) before use. The water content of clean sediment after the treatment above was 43.2% (± 0.5%; n = 3), determined by drying for 12 h at 105°C, and total organic matter content was 2.7% (± 0.3%; n = 3), determined by weight loss on ignition for 6 h at 550°C.

Two types of sediment exposures with Cu were prepared. First, sediments were amended with Cu in 3 forms: aqueous ions ($CuCl_2 \cdot 2H_2O$, Sigma–Aldrich, Denmark), 100 nm NPs and micron-sized particles. Stock solutions of aqueous ions and stock dispersions of 100 nm NPs and micron-sized particles were prepared at a concentration of 20 mg Cu/L in MilliQ water, which was mixed into clean sediment to a nominal concentration of 250 µg Cu/g dry wt sed. Cu-amended sediment was homogenized on a shaking table for 24 h.

Second, sediments were amended using the same procedure as above but with CuO NPs of 3 different shapes: spheres, spindles, and rods. The CuO NP suspensions were 2605 mg/L for spheres, 4243 mg/L for spindles and 1878 mg/L for rods. A known volume of suspension was transferred into clean sediment to prepare amended

sediment at a nominal concentration of 250 µg Cu/g dry wt sed. In addition, a control group (no extra addition of Cu) was prepared.

2.3 Organisms

The *Capitella teleta* used in this study were derived from laboratory cultures of worms that were originally collected in Setauket Harbour, New York, USA and identified as *Capitella* sp. I by J. Grassle (personal communication). Worms were cultured in sieved, field-collected sediment (2 to 5 cm layer, grain size $\leq$ 250 µm) and seawater (31‰ salinity) at 17°C. In order to ensure that all exposed worms were of the same life stage, eggs were collected from brood tubes in the culture approximately 14 d before exposure. Hatched larvae in brooding tubes were transferred into glass beakers with clean sediment (≤ 125 µm), 100 ml seawater (31‰ S) and air pumps. The initial average body size of worms was 1.2 ± 0.4 mm^3 (n = 70) measured from randomly selected juvenile worms (see section *2.6*).

2.4 Experiment setup

The experiment started with 7 days exposure in amended sediments followed by another 7 d of depuration in clean sediments. One day before exposure, worms were transferred to clean seawater for 24 h. On exposure day 1, individual worms were transferred into multi-wells (one worm per well) with 1.5 ml sediment and 2 ml seawater (6.5 ml well, approximately 3 mm height of sediment). Overlying seawater was changed with fresh, clean aerated seawater twice a week. In total, 100 replicates were prepared for exposure for each treatment. During 7 d of exposure, 10 replicate individuals were sampled from each treatment on day 1, 3, 4, 5 and 7. The remaining worms (i.e., 50 replicates/treatment) were transferred into clean sediment for 7 d of depuration. During the depuration phase, 10 replicates were sampled on day 2, 3, 4, 5 and 7. Sampled worms were allowed to empty their guts of sediment in clean

seawater for 24 h, their body volumes were measured by image analysis (see section 2.6, and their Cu body burdens measured by atomic absorption spectrometry (AAS) (see section *2.7*).

2.5 Body volumes

On each sampling day, survival worms were transferred into clean sediment and empty sediments in their guts overnight. They were videotaped with a video camera mounted on a dissecting microscope. Body length (L, mm) and surface area (A, mm^2) were measured using an image-analysis program (SigmaScan Pro software, ver. 5.0.0., SPSS®). Assuming that worms are cylindrical in shape, initial body volumes (BV, mm^3) were estimated as $BV = \pi A^2/4L$ (Self and Jumars, 1978).

2.7 AAS measurement

Flame AAS was used to measure total Cu concentrations in sediment at the beginning of the experiment whereas graphite AAS was used to measure accumulated Cu concentrations in worms. The AAS (Spectra AA-220, Varian, Australia), and the sample digestion procedure were as the same as those used by Pang et al. (2012). In brief, after being lyophilized (Christ Alpha 1-2, Osterode, Germany) at -50℃ overnight, all samples (both sediment and worms) were digested with 65% HNO_3 in a microwave oven (Milestone MLS-1200 Mega, Leutenkirch, Germany) by a program of 6 min at 250 W, 400 W, 650 W and 250 W. The acid digestion were filtered and measured on AAS. Two to four worms were pooled together for graphite AAS measurement and a minimum of 2 analytical replicates were made for each treatment at each sampling time.

2.8 Uptake and depuration kinetics of Cu

The net uptake rate constant (k_u) obtained during the exposure period was calculated following Spacie and Hamelink (1985) as the mean value of measuring tangents of

accumulated Cu in exposed worms at each sampling time ($\Delta C/\Delta t$) applied to the equation: $ku = (\Delta C/\Delta t + kd \times C)/Csed$. *C* is the weight-specific body burden of Cu (WSBB, µg Cu/g dry wt worm,) at each sampling d during the exposure period and C_{sed} is the measured Cu concentration in the sediment at the beginning of the exposure period (Spacie and Hamelink, 1985). The depuration rate constant (k_d) was calculated as the slope of the linear regression between the Ln-log WSBB ($\ln C$) and sampling time, based on a one-compartment depuration model as follows - $\ln C(i) = \ln C(0) - kd \times t$. $\ln C(0)$ and $\ln C(i)$ are Ln WSBB on day 0 and days 2, 3, 4, 5 or 7 of the depuration period. Half-life (t½, time to 50% reduction in body burden) was calculated as $t½ = \ln 2/kd$.

2.9 Statistics

A contingency analysis using the Chi-square method was used to test whether the percentage of surviving worms at the end of exposure and at the end of depuration was dependent on treatment. Non-parametric Kruskal-Wallis tests or analysis of variance (ANOVA) were used to test effects of Cu form and CuO NP shape on BVs and WSBB after exposure and depuration, respectively, depending on whether the data fulfilled the assumptions of ANOVA. Multi-slope comparisons (F [k, *d*f]) were made on k_d to detect effects of Cu form and CuO NP shape based on comparison of simple linear regression by Zar (1999). Tukey's test was used to compare endpoints difference between any two treatments. Statistics are presented as p values unless otherwise stated. Groups were defined as significantly different if $p \leq 0.05$ and marginally significant if $0.05 < p < 0.10$.

3. RESULTS

3.1 Measured Cu concentrations

The initial sediment Cu concentrations in the different treatments were 22.8 ± 0.8 µg Cu/g dry wt sed (control), 246.7 ± 13.1 µg Cu/g dry wt sed (aqueous ions), 243.7 ± 31.8 µg Cu/ g dry wt sed (100 nm NPs), 224.9 ± 14.8 µg Cu/g dry wt sed (micron-sized CuO particles), 225.7 ± 3.5 µg Cu/g dry wt sed (CuO spheres), 228.6 ± 3.1 µg Cu/g dry wt sed (CuO spindles), and 262.6 ± 26.7 µg Cu/g dry wt sed (CuO rods) (n = 3). There were no significant differences in measured sediment Cu concentrations among treatments (without control, all Cu forms and NP shapes) at the start of the experiment (One-way ANOVA, $p = 0.138$).

3.2 Survival

More than 90% of the worms survived after 7 d of exposure (**Table 1**). During the exposure period, no significant effects (all Cu treatments and control) were detected on worm survival (Chi-Square test, $p = 0.477$), indicating that none of the Cu exposures were lethal for *C. telata*. During depuration, significant effects of Cu exposure were detected on worm survival (Chi-Square test, $p = 0.005$) compared to control worms, but no significant differences were detected among Cu forms or shapes (Chi-Square, $p = 0.566$).

Comparing worm survival within treatments, significant decreases in survival of worms were found in all treatments except in the control group between the exposure period and depuration period (Chi-Square test, $p = 1.000$ for control but $p < 0.001$ for all Cu treatments). The survival decreased by approximately 23% on average in all Cu treatments (pooled data) during the depuration indicating a delayed effect due to Cu exposure in all cases.

3.3 Body volumes

Worms in all treatments grew during the exposure and depuration periods (**Fig. 1**). At the end of the exposure period, BVs on day 7 were not significantly affected by Cu in

any form (One-way ANOVA, $p = 0.166$, $n = 10$ with 4 treatments incl. control) or CuO NP shape (Kruskal-Wallis test, $p = 0.622$). The average BV increased from 1.2 mm^3 to 3.5 mm^3 during 7 days of exposure (pooled data for all Cu treatments and control) corresponding to a growth rate of 0.153 d^{-1}. The same was true at the end of depuration period, with no significant effects of Cu in any form (One-way ANOVA, $p = 0.137$, $n = 10$ with 4 treatments incl. control) or CuO NP shape (One-way ANOVA, $p = 0.666$) on BV on day 7. The average BV increased from 3.5 mm^3 to 5.5 mm^3 during the 7 day depuration period (pooled data) corresponding to a growth rate of 0.056 d^{-1}.

3.4 Weight-specific body burden

Compared to worms in control, the increase of WSBB was approximately 5-fold for the aqueous ions treatment, 4-fold for 100 nm NPs, 10-fold for micron-sized CuO particles, 4-fold for spheres, 11-fold for spindles and 18-fold for rods at the end of the exposure period indicating that all Cu forms and shapes were bioavailable to *C. teleta* (**Fig. 2**). These observed differences were statistically significant as follows: significant effects of Cu form were detected (ANOVA, $p = 0.039$-incl. control, ion, 100nm and micron); post-hoc pairwise comparisons detected a significant difference between micron-sized particles and control (Tukey's test, $p = 0.029$) but not between the aqueous ion treatment and the control or between 100 nm NPs and the control (Tukey's test, both $p > 0.050$). Furthermore, there was no significant differences among treatments of aqueous ions, 100 nm NPs and micron (Turkey's test, $p > 0.05$). Significant effects of CuO NP shape were detected (One-way ANOVA, $p = 0.001$), with WSBBs of control and spheres treatments being significantly lower than those of spindles or rods treatments (Tukey's test, spheres vs. spindles: $p = 0.078$, spheres vs.

rods: p = 0.003, control vs. spheres: p = 0.505 and control vs. spindles p= 0.018, control vs. rods: p = 0.001).

At the end of the depuration period, WSBB in the Cu treatments was at the same level as in the control, and there were no significant effects of Cu form (Kruskal-Wallis test, p = 0.277) or CuO NP shape (Kruskal-Wallis test, p = 0.172).

3.5 uptake and depuration kinetics of Cu in Capitella teleta

Net uptake rates of Cu (k_u) in various forms and CuO NPs in different shapes are shown in **Table 2**. Uptake rates for the aqueous ions treatment, 100 nm NPs, and spheres were similar and approximately 8-10% d^{-1}. The fastest net uptake rate was observed for the spindles treatment (39% d^{-1}), followed by rods (28% d^{-1}) and then micron-sized particles (19% d^{-1}). There was no indication of WSBB approaching a steady state in any treatment during exposure period of 7 d.

Accumulated Cu from amended sediments was depurated significantly after worms were transferred to clean sediment for 7 d, except for the 100 nm NP treatment, which showed marginal depuration, and for the control which showed no depuration (**Table 3**). There were no significant differences in depuration rates (k_d) as a function of Cu form (Multi-slope comparison, F [3, 33] = 0.778) or CuO NP shape (F [3, 34] = 0.370). More than 80% of the accumulated Cu was lost during 7 d in clean sediment indicating that the accumulated Cu was readily eliminated in all cases.

4. DISCUSSION

4.1 Effects

Cu is one of essential elements to support physiological requirements in organisms, it is toxic to aquatic organisms at high levels. Ionic Cu may cause damages to aquatic organism by interfering with osmoregulation, inhibiting respiratory enzymes and reducing the activities of regulatory enzymes of ATP synthesizing pathways (Hodson

et al., 1979, Hubschman, 1967, Hansen et al., 1992). The death of exposed organisms to Cu was caused by tissue hypoxia associated with reduced enzyme metabolisms (Hansen et al., 1992), which may also be the reason for the toxicity of CuO NPs. The decreased abundance in *C. teleta* was observed in a highly polluted location with Cu concentration up to 200 µg/g sed (Brage, 1985). In the present study no toxicity of Cu in different forms or CuO NPs' shape was observed during the exposure period in any treatment with Cu at a comparative sediment concentration of Cu in Rygg (Brage, 1985) but survival was decreased by approximately 23% during the subsequent depuration period. This delayed toxicity of Cu was possibly caused by the short exposure period of 7 days in the present study. Toxicity was displayed usually when accumulated Cu was more than its threshold body burden level which was determined by both total sediment concentration of Cu and exposure time (Rainbow, 2007). The threshold body burden of Cu was unclear in *C. teleta* yet. In a comparative species (*Neanthes arenaceodentata*) exposed to 0.1 mg Cu/L seawater, the time to 50% mortality was longer than 36.5 days and Cu body burden in dead worms was approximately 1000 µg/g dw worm (Pesch, 1979). For *C. capitata*, the Cu concentration to cause 50% mortality after 96 h exposure was 0.02-0.2 mg Cu/L seawater (Reish and Gerlinger, 1997). According to the equilibrium partitioning model of Cu in sediment compartment, Cu concentration in pore water of sediment was 12.62 – 0.50 µg/L in the present study (Feng et al., 1999) which was lower than LC_{50} after 96 h in Reish and Gerlinger (1997).

In the present study, the average growth rate was 0.153 d^{-1} (15.3% d^{-1}) and 0.065 d^{-1} (6.5% d^{-1}) including the control treatment during both exposure and depuration periods, respectively. The lower mean growth rate during the depuration period than the exposure period is likely due to a decline in growth rate with increasing worm size

(Ramskov and Forbes, 2008). It is well known that growth rates of the species differ in sediment with different organic matter quantity and quality. Linton and Taghon (2000) reported growth rates of the same species decreased from approximately 23% d^{-1} to less than 10% d^{-1} with the reduction of organic matter quantified as total carbon and total nitrogen concentrations in tested sediment. In the present study, although the total organic matter content of 2.7% was close to the highest total organic matter content in Ramskov and Forbes (2008), it was not clear for the quality of sediment used. The quality of sediment was referred by organic carbon content, organic nitrogen content, amino acid, essential fatty acid content and the amount of labile organic matter (Tenore, 1977a, Tenore, 1977b, Taghon, 1981, Cheng et al., 1993). For instance, *C. teleta* grew faster in population feeding on mixed cereal with high quality than on marsh grass with low quality (Tenore, 1977a). Therefore, a relative low growth rate of *C. teleta* may due to the low organic matter quality of the sediment used in the present study.

4.2 Bioaccumulation

The bioaccumulation and the net uptake rate of Cu was a consequence of uptake from all environmental sources (i.e., water and sediment) and depuration in *C. teleta*. That positive net uptake rate constants indicated that Cu in all forms and CuO NPs in all sizes and shapes in the present study have the potential for bioaccumulation in *C. teleta*. No dissolution of similar CuO NPs as used (i.e., 100 nm from Intrinsiq Materials Limited) has been detected in marine sediment by diffusive gradients in thin films (Buffet et al., 2011). Otherwise, the net uptake rate of CuO particles would be overestimated if Cu ions were released from CuO NPs.

The observed similar net uptake rates of Cu administered in different forms suggest that Cu uptake from CuO particles was independent on Cu form. For the different

CuO NP shapes, uptake rate constants was higher from sediment amended with spindles and rods compared to spheres. Uptake of Cu ions involves transportation across the cell membrane through available protein carriers (Luoma, 1983); while uptake of NPs occurs via endocytosis (Conner and Schmid, 2003). Endocytosis rates of single-walled carbon nanotubes (50 nm) and gold nanoparticles (50 nm) have been determined to be 10^{-3} min^{-1} (equal to 1.440 d^{-1}) and 10^{-6} min^{-1} (equal to 0.001 d^{-1}), respectively (Wang et al., 2010), which is comparable to the rates measured in the present study (i.e., 0.104 d^{-1} for 100 nm CuO NPs; 0.192 d^{-1} for micron CuO paticles; 0.082 d^{-1} for spheres; 0.394 for spindles and 0.283 d^{-1} for rods). In addition, different endocytotic pathways and uptake rates have been displayed in direct internalization of nanoparticles in different cells. Positive charged nanoparticles are preferentially internalized into in HeLa (cervical cancer cell line) cells by the clathrin-mediated endocytotic pathway at a higher uptake rate than negative charged nanoparticles (Harush-Frenkel et al., 2007). Uptake rate of negative charged cerium oxide nanoparticles was found higher than positive charged nanoparticles in adenocarcinoma lung cells (Patil et al., 2007). In a study of cellular internalization pathways of nanoparticles by Gratton et al. (2008) found that lager particles of 50-100 nm were internalized through the caveolae-mediated endocytotic pathway. Thus, different uptake pathways and cell lines may be involved in the internalization of CuO NPs and micron CuO particles in the present study.

The effect of particle shape has not been fully understood on the uptake of NPs yet. Gold spheres (14 nm and 74 nm) were taken up approximately 3 times more than gold rods (14 nm × 74 nm) into HeLa cells after 6 hours (Chithrani et al., 2006). On the contrary, Gratton et al. (2008) found a higher uptake rate of poly-organic nanoparticles in rods (150 nm × 450 nm and 100 nm × 300 nm and), followed by

cubes (200 nm × 200 nm) and spheres (1 μm, 2 μm, 3 μm and 5 μm) in HeLa cells. It was suspected that more receptors on cell surface interacted with the long axis of rods facilitating their uptake (Hutter et al., 2010). This may also be the case for the uptake of CuO NPs in spindles, rods and spheres in Paper III that the orientation of the long axis facilitates their internalization into *C. teleta*.

After 7 days of depuration, the body burden of Cu was decreased to the same background level as the control worms regardless of Cu treatment. These results suggest that C. *teleta* can eliminated Cu regardless of Cu form or nanoparticle shape taken up. Depuration of accumulated Cu ions involves that intracellular Cu is sequestered in cytosolic proteins (i.e., metallothioneins/-like proteins) and transported to lysosomes for degradation into insoluble residual bodies followed by excretion (Amiard et al., 2006, Wang and Rainbow, 2010). It is not clear what depuration mechanisms are involved in depuration of Cu NPs. Accumulated Cu may be eliminated both as particles and ions. However, it is not possible to distinguish between the two using the one-compartment model which was used in the present study. Nevertheless, the high elimination rate constants (k_ds) of spindles and rods suggest that they are eliminated mostly as particles. The 'Trojan horse' concept has, in addition to the toxicity aspect, also been proposed for the elimination of ENPs, in which the discrete metal ions delivered by ENPs into tissues are transported and eliminated through the existing cellular processes (Park et al., 2010). For instance, Ag accumulated from Ag NPs was eliminated in two compartments, that Ag NPs were eliminated at a higher rate in first 4 days followed by elimination of discrete Ag ions at a slower rate in deposit feeding invertebrate, *P. ulvae* (Khan et al., 2012). This may also be the case for depuration of Ag NPs because the presence of surfactants and proteinaceous materials in digestive fluid may facilitate Ag ion released from

accumulated Ag NPs in polychaete, *C. teleta*. It has been reported that the desorption of Cu ions from ingested sediment were increased in the presence of histidine, proteinaceous materials (studied on its surrogate, bovine serum albumin) and sodium taurocholate (present in surfactants micelles) in digestive fluid of polychaetes (Zhong et al., 2012, Pettibone et al., 2008, Jones and Turner, 2010). In addition, Golobic et al. (2012) found that 99% Cu accumulated from food (common hazel leaves) spiked with Cu NPs was present as Cu ions in the isopod, *Porcellio scaber*. Therefore, depuration of released metal ions can be eliminated from the depuration of ENPs in *C. teleta*. However, more studies should be undertaken to understand the dissolution of CuO particles in the gut of *C. teleta* and its role in bioavailability of ENPs.

5. CONCLUSION

The low mortality of *C. teleta* may due to short exposure period of 7 days to Cu in aqueous ion, nano (several shapes) and micron forms in sediment at concentrations of approximately 200 µg/g dw sed. All worms grew positively in Cu treatments. All Cu treatments (aqueous ion, NP and micron forms and CuO NPs in different shapes) were bioavailable and bioaccumulated in *C. teleta*. There were no form- or particle shape-dependent net uptake rates was observed and no difficulty was observed in depuration of accumulated Cu from different treatments. Uncertainties remain with regard to the behaviour of nanoparticles in sediments, and internalization and cellular dissolution of CuO NPs and micron particles in *C. teleta*.

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Acknowledgements

This work was supported by Roskilde University (RUC) and is done in cooperation with the EU NanoReTox project (7th Framework Programme, Grant Agreement no.

CP-FP214478-2). The authors gratefully acknowledge to technicians (Anne-Grethe Winding, Michael Rabe and Anne Faarborg) working in the Ecotoxicology Lab for their technical guidance and assistance during experiments.

FIGURE CAPTIONS

Fig. 1. Body volumes of worms over time after (a) exposure to Cu in various forms; (b) exposure to CuO nanoparticles (NPs) in different shapes; (c) depuration of Cu in various forms, and (d) depuration of CuO NPs in different shapes ($n > 9$ for exposure period and $n > 6$ for depuration period). Note, the data from the control and aqueous ion treatments are present on both graphs (a, b and c, d, respectively) to provide a common reference. Error bars refer to ± 1 standard deviation.

Fig. 2. Weight-specific body burden of Cu in worms over time after (a) exposure to Cu in various forms; (b) depuration of Cu in various forms; (c) exposure to CuO nanoparticles (NPs) in different shapes, and (d) depuration of CuO NPs in different shapes ($n = 3$). Note, the data from the control and aqueous ion treatments are present on both graphs (a, b and c, d, respectively) to provide a common reference. Error bars refer to ± 1 standard deviation. '*' refers to significant difference between control and micron in (a) and between spheres and rods in (c); '+' refers to significant difference between control and spindles and between control and rods in (c).

TABLE

Table 1. Mortality frequency of *C. teleta* in different Cu treatments after the 7 days exposure period and the 14 days exposure and depuration period.

Cu treatment	Exposure		Exposure and depuration	
	Total worm	Dead worm	Total worm	Dead worm
Control	50	0	50	0
Aqueous ions	50	2	50	14
100 nm NPs	50	4	50	13
Micron	50	1	50	15
Spheres	50	2	50	17
Spindles	50	1	50	9
Rods	50	2	50	11

Table 2. Net uptake rates (k_u, d^{-1}) for *Capitella teleta* in different Cu treatments (n=3).

Exposure day	Control	Aqueous ions	100 nm NP	Micron	Spheres	Spindles	Rods
0	-	-	-	-	-	-	-
1	0.090	0.043	0.103	0.061	-0.056	0.084	0.163
3	0.143	0.046	0.078	-0.013	0.080	0.114	0.179
4	-0.003	0.064	-0.146	0.295	0.094	0.161	0.180
5	0.008	0.198	0.657	0.415	0.212	1.845	0.278
7	0.051	0.071	-0.174	0.204	0.077	-0.234	0.615
Average k_u	0.058	0.084	0.104	0.192	0.082	0.394	0.283

Table 3. Depuration rates (k_d, d^{-1}) and half-life (t½, d) of accumulated Cu for *Capitella teleta* (n=3) in different treatments.

Treatment	k_d	p	R^2	t½
Control	-0.133	0.169	0.222	-
Aqueous ions	-0.271	0.007	0.568	3
100nm NP	-0.148	0.058	0.249	5
Micron	-0.283	0.048	0.369	2
Spheres	-0.332	0.005	0.696	2
Spindles	-0.363	0.000	0.737	2
Rods	-0.422	0.000	0.688	2

FIGURE

Fig. 1

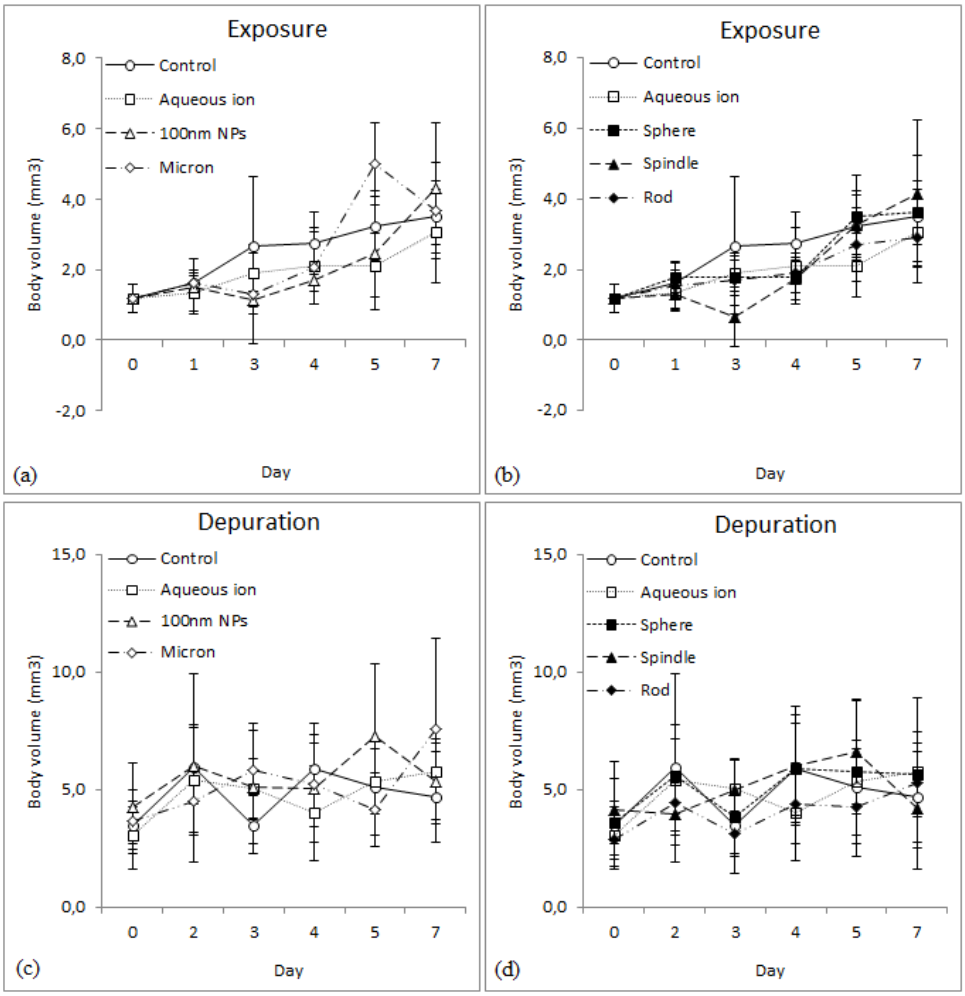
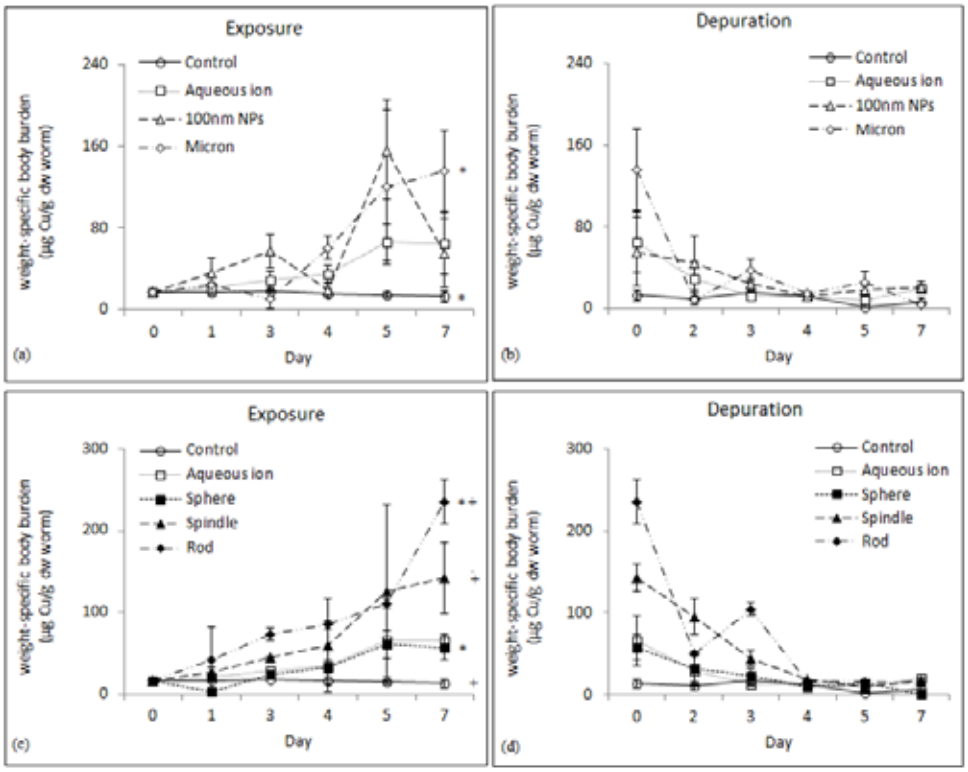

Exposure
Control
Aqueous ion
100nm NPs
Micron
Body volume (mm3)
8,0
6,0
4,0
2,0
0,0
-2,0
0
1
3
4
5
7
(a)
Day
Exposure
Control
Aqueous ion
Sphere
Spindle
Rod
Body volume (mm3)
(b)
Day
Depuration
Control
Aqueous ion
100nm NPs
Micron
Body volume (mm3)
15,0
10,0
5,0
0,0
0
2
3
4
5
7
(c)
Day
Depuration
Control
Aqueous ion
Sphere
Spindle
Rod
Body volume (mm3)
(d)
Day

Fig. 2


Exposure
Depuration
Control
Aqueous ion
100nm NPs
Micron
Sphere
Spindle
Rod
weight-specific body burden (µg Cu/g dw worm)
Day
(a)
(b)
(c)
(d)

Paper IV

Effects and biokinetics of silver-and copper-bearing nano-particles in a marine deposit feeder, *Macoma balthic*a

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Aqautic Toxicology. Submitted

I have ocntributed to this paper by:
Taking part in the development of the idea and design of the experiment
Taking part in investigating the experimental work
Writing the manuscirpt

Effects and biokinetics of silver-and copper nanoparticles in a marine deposit feeder, *Macoma balthic*a

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Abstract

Silver and copper oxide nanoparticles (NPs) have wide applications in industry and commercial products and they are released from e.g., wastewater into the aquatic environment. Limited information is currently available on metal NP effects, bioavailability and biokinetics in marine bivalves. In the present study, a deposit feeding clam, *Macoma balthica* was exposed to sediment amended with Ag and Cu in different forms (aqueous ions, nanoparticles and micron-sized particles) in 3 experiments. Two sizes (20 and 80 nm) of Ag and one size (100 nm) of CuO nanoparticles were tested. In all experiments, no effects on mortality, condition index or burrowing behavior were observed for any of the metal forms at the concentrations tested (150-200 μg g^{-1}), during 35 d exposure. No genotoxicity was observed at the end of the experiments, measured as DNA damage with the single-cell gel electrophoresis assay (Comet Assay). Bioaccumulation of both Ag and Cu in the clams was form depend following: aqueous ion > nanoparticles > micron-sized particles. Cu uptake and depuration kinetics were studied in more detail yielding uptake rates (k_u, d^{-1}) in soft tissue of 0.640, 0.464 and 0.091 for sediment amended with Aqueous Cu ions, CuO nanoparticles and micron-sized CuO particles, respectively, supporting that net uptake was dependent on form. Depuration rates (k_d, d^{-1}) from soft tissue were -0.074, -0.030 and 0.019 for Aqueous Cu ion, CuO nanoparticle and micron-sized CuO particle, respectively. Higher Cu bioaccumulation in smaller clams than in larger clams suggested that more biological factors such as clam size, food availability and clam origin habitat, etc. should be taken into account with bioaccumulation of nanoparticles in further experiments.

Key words: *Macoma balthica*, bioaccumulation, biokinetics, nanoparticles, metallic form, Comet Assay

1. INTRODUCTION

Engineered nanoparticles (ENPs) have been produced on a large scale and have been applied widely in commercial products such as clothing, toiletries and food storage containers for several years (Klaine et al., 2008, Benn and Westerhoff, 2008). ENPs refer to metal particles with at least one dimension between 1 nm and 100 nm intentionally (US EPA). Due to the reduction of particle size down to the nanoscale, ENPs have specific properties due to increased surface to volume ratio and particle-quantum effects (Ju-Nam and Lead, 2008). It has been demonstrated that ENPs and corresponding metal ions released from ENPs enter aquatic environments via sewage treatment plant (Gottschalk et al., 2009). Sediments are the ultimate sink of most metals including ENPs, and deposit feeders in particular may thus be at high risk of exposure to ENPs.

Recently, toxicity and bioaccumulation of CuO NPs has been found in several deposit feeders. For instance, Pang et al. (2012) found CuO NPs mixed into sediment caused adverse effects on growth, feeding rate and reproduction in the freshwater snail, *Potamopyrgus antipodarum*. CuO NPs also induced superoxide dismutase-derived oxidative stress activity and were observed in digestive glands of *Mytilus galloprovincialis* (Gomes et al., 2012). Burrowing activity was affected in two deposit feeders, *Scrobicularia plana* and *Hediste (Nereis) diversicolor* exposed to sediment amended with Ag NPs and CuO NPs (Buffet et al., 2011, Cong, 2011). In addition, DNA damage of Ag NPs was also detected *N. diversicolor* (Cong et al., 2011). Although the bioaccumulation of ENPs has been reported in several species (*N. diversicolor*, *S. plana, Perna viridis*, *Lymnaea stagnails* and *Peringia ulvae*) (Cong et al., 2011, Pan et al., 2012, Croteau et al., 2011, Buffet et al., 2011, Khan et al., 2012), biokinetics of ENPs are still not fully understood. The 'Torja horse' concept proposed that nanoparticles deliver bundles of metal ions into the tissue of organisms (Limbach et al., 2007), which is observed by a two-phase depuration of Ag NPs in *P. ulvae* and *L. stagnails* (Khan et al., 2012). To our knowledge no other ENPs or species has been studies in uptake and depuration kinetics yet.

Macoma balthica is one of the most common and numerous macrobenthic species in estuarine and marine habitats such as the Baltic Sea (Beukema and De Bruin, 1977), and is an important food source for flatfish and wading birds. *M. balthica* is a filter feeder in sandy sediment and a deposit feeder in muddy sediment (Olafsson, 1989). Thus, *M. balthica* is a potential organism for exposure to ENPs in sediments and play an important role in marine food web.

The goal of the present study was to investigate the bioaccumulation and effects of sediment-associated Ag and CuO nanoparticles in *M. balthica* in 3 interrelated experiments. The two metals were amended to sediment as Ag^{+} and Cu^{2+} (i.e., aqueous ions), as Ag^{0} and CuO nanoparticles, and as micron-sized particles. The effects of metal form on the clam were evaluated both at the organism level (mortality, condition health and behavior) and at sub-organism level (DNA damage by Comet Assay). Furthermore, the biokinetics parameters (k_u and k_d) were quantified to examine the bioaccumulation pattern of the tested Cu forms.

2. METHODS

Three experiments were set up in the present study. Experiment 1 tested effects and bioavailability of Ag in different forms; Experiment 2 tested effects and uptake of Cu in different forms and Experiment 3 assessed the depuration of Cu in the same forms as in Experiment 2. *M. balthica* was collected from different sites of the Isefjorden in the summer, 2010, and winter, 2011 for Ag and Cu exposures, respectively (Fig. 1). Before exposure, clams were kept in clean water (16‰) for two weeks.

All sediment used in the experiments was collected from the Isefjorden (55°40'N, 11°47'E, Munkholm, Denmark) and sieved through 500 μm mesh with deionized water and rinsed twice with seawater (16‰) prior to experimental initiation. Sediment was spiked with a nominal concentration of 200 μg metal/g dry weight sediment (dw sed, both Ag and Cu) after being frozen at -20˚C for several weeks. The organic matter content of sediments was determined on ignition at 550 °C for 6 hours after spiking. Afterwards, 300 g wet weight (ww) spiked sediments or control sediments (i.e., approximately 4 cm height) was transferred into plastic beakers (500 ml, 7 cm

diameter). Seawater (200 ml) was added to each beaker. After 24 h of equilibration between seawater and sediment at 17˚C, overlying seawater was changed before addition of animals.

2.1 Experiment 1: Silver

2.1.1 sediment treatment

Sediments were amended with Ag in 3 forms and NPs with 2 sizes. The Ag forms added were Aqueous Ag ion ($AgNO_3$, Sigma-Aldrich, USA), AgNPs-20 and AgNPs-80 (20 nm and 80 nm particles in powder, with w/~0.3% PVP coating, Nanostructured & Amorphous Materials Inc., USA) and Micron Ag (2 to 3.5µm Ag particles in powder, Sigma-Aldrich, USA). These particles were from the same batch used in Cong (2011). Hydrodynamic diameters were 187 nm and 144 nm for AgNPs-20 and AgNPs-80, respectively. Zeta potentials were -39.4 mV and -42.3 mV for AgNPs-20 and AgNPs-80, respectively. Micron-Ag had a wide size distribution from 8 nm to 3 µm and zeta potential of -49.0 mV (Cong et al., 2011). In addition, stock solution (Aqueous Ag ion) and stock dispersion (AgNPs-20, AgNPs-80 and Micron-Ag) was amended to sediments as described by as Cong et al. (Cong et al., 2011). Briefly, a prepared stock solution/dispersion of Ag in different forms was prepared in MilliQ water. The preparation included 15 mins ultrasonicated (80 W, 45 kHz) in a water bath (Ultrasonic Bath VWR, Lutterworth, UK) followed by a 15 min pause. This process was repeated 4 times. Subsequently, a given volume stock solution was transferred into clean sediment to provide a nominal concentration of 200 µg/g dry weight sediment (dw sed). In addition one control group (clean sed) and one PVP control group (0.3% weight of AgNPs amount in amended sediment mimicking the concentration of the coating in the NP exposure group) were included. All sediments were homogenized thoroughly with a metal spoon for several times during 24 h before exposure.

2.1.2 Experimental setup

The experiment consisted of 6 treatments each with 5 replicates (i.e., a total of 30 clams). Exposure time was 5 weeks. Initial shell length was 11.0 mm (± 2.0 mm,

n=120) measured by an image-analysis program (SigmaScan Pro software, ver. 5.0.0., SPSS, Chicago, IL, USA) in anterior-posterior direction of the clam, and the organism wet weight was 391.0 mg on average (±234.1 mg, n = 120).

On the first exposure day the overlying seawater in each beaker was replaced with pre-aerated, clean seawater. Sediments in each beaker were then allowed to settle for 1 hour before clams were added. Clams collected from different sites were grouped randomly (4 per beaker) and added to the beakers at the same time. Unburied clams were replaced after 24 h. All containers were supplied with an air pump, covered with parafilm and keptat 15°C in darkness throughout the experiment. After 35 d exposure without additional food, all living clams were transferred into pre-aerated, clean seawater for 24 h of depuration to ensure that they had emptied their gut. Afterwards, clams were dissected into soft tissue and shells and stored at -20°C for atomic absorption spectrometer (AAS) measurements.

2.2 Experiment 2: Copper

2.2.1 sediment treatment

Sediments were amended with Cu in 3 forms: Aqueous Cu ions ($CuCl_2 \cdot 2H_2O$, Sigma Aldrich, Denmark), CuO NPs in powder (<100 nm, NHM, UK) and Micron-CuO in powder (<5 μm, cat. #20, 884-1, assay 98%, Sigma–Aldrich, Denmark). CuO NPs and Micron-CuO were from the same batch as used in Pang (2011) and Pang et al. (2012). CuO NPs were poly-dispersed with a hydrodynamic size of 204 nm and a zeta potential of +42 mV. Particles on nano scale and on micron scale were co-existed in Micron-CuO which had a zeta potential of -16.9 mV. 10 mg Cu/L MilliQ water stock solution (Aqueous Cu ion) and stock dispersion (CuO NPs and Micron-CuO) were added to sediments to achieve a nominal concentration of 200 μg/g dw sed. In addition, a control group (no addition of Cu in any form) was also treated similar to the Cu treatments. All exposed sediments were homogenized thoroughly by hand before starting exposure.

2.2.2 Experiment setup

M. balthica was exposed to one of 3 treatments of Cu amended sediment or a control

treatment for 5 weeks. Each treatment consisted of 6 replicates with 42 clams in total. Initial shell length was 9.6 mm (±1.4 mm, n=168) measured in the same way as Experiment 1.

On the exposure day, mixed clams collected from different sites were randomly grouped (6 per beaker) and added into beakers at the same time. Unburied clams were removed after 24 h. All containers were fitted with air pumps, covered with parafilm and kept at 15°C in darkness during the exposure. On day 3, 7, 11, 15, 19, 23, 27, 31 and 35, an extra food source, the cryptophytic algae *Rhodomonas salina,* was provided at a concentration of $2x10^4$ cells/ml to the overlying water for 1 h. Afterwards, 200 ml overlying water was changed with clean seawater (16‰). After feeding, one replicate with 7 individuals was sampled on day 3, 7, 11, 15, 23 and 35. Sampled clams were transferred into clean seawater for another 24 h of depuration. Then, they were dissected into soft tissue and shells and stored at -20°C for atomic absorption spectrometer (AAS) measurements.

2.3 Experiment 3 Cu

2.3.1 Amended sediment

Amended or clean sediment used for exposure in Experiment 3 was as same as in Experiment 2.

2.3.2 Experiment setup

Initial shell length was 12.0 mm (±1.0 mm, n=168) and clams were treated in the exact way of Experiment 2 including sediment (with and without Cu), seawater, air pump, parafilm, extra food, feeding time and water changing. After 35 days exposure, four individuals of each treatment were prepared for both AAS measurement and Comet Assay. The rest of clams were transferred into clean sediment immediately to depurate accumulated Cu for 15 days. One or two individuals from each treatment were sampled on each day during the depuration phase. Sampled clams were transferred into clean seawater for a 24-h depuration before AAS measurements.

2.4 Burrowing behavior

The burrowing behavior of clams was evaluated when first added into both control

and Cu amended sediments. Unburied clams were noted for each treatment during the first 24 h, and they were removed after 24 h. The burrowing activity was calculated as the percentage of unburied clams calibrated with total clams left after 24 h.

2.5 Condition index

Condition index (CI) was calculated on day 35 of Experiment 1 and on each sampling day of Experiment 2. Since measuring the CI required sacrificing clams, CI wasn't measured in Experiment 3 after Cu exposure. It was calculated as a ratio of soft tissue dw (mg) to shell length (mm).

2.6 Bio-kinetics of Cu in various forms

Uptake rate constants (k_u) were obtained from Experiment 2. They were calculated as the mean value of measuring tangents of Cu concentrations in soft tissue or shell, to the uptake curve between each sampling time ($\frac{\Delta C}{\Delta t}$) following Spacie and Hamelink (1985): $k_u=(\Delta C/\Delta t+k_d \times C)/C_{sed}$, where C was measured as Cu concentration in soft tissue or shell on each sampling day and C_{sed} was measured Cu concentration in amended sediment at the beginning of the experiment (Spacie and Hamelink, 1985). Depuration rate constant (k_d) estimates were obtained from Experiment 3. They were calculated as the slope of the linear regression of the natural log transformed Cu concentrations in soft tissue or shell (C_{tissue} or C_{shell}, after exposure to Cu amended sediments) versus time (t, 15 days depuration) for clams depurating in clean sediment. A simple, one-compartment depuration model was assumed: $ln\ C_{tissue} = ln\ C_0 - k_d\ x\ t$. Half-life (t½, time to 50% reduction in body burden) was calculated as $t½ = ln\ 2/k_d$.

2.7 Comet Assay

Genotoxicity of Cu was assessed in Experiment 3. The assessment was done by quantifying strand breaks on the DNA using the single-cell gel electrophoresis assay, based on a modification of the procedure described by (Rank et al., 2005). Agarose solutions were dissolved in Kenny's salt solution (200 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, and 2 mM $NaHCO_3$). Upon experimental termination the clams were opened by cutting the anterior and posterior adductor muscles, and the soft tissues transferred into glass beakers. All work was carried out in yellow light afterwards, in

order to prevent UV damage to the DNA. The soft tissues were washed four times with 2ml calcium-magnesium-free saline (CMFS) consisting of 20 mM Hepes, 300 mM NaCl, 12.5 mM KCl, 5 mM EDTA. The tissues were subsequently cut with scissors for 30 sec in a 2 ml CMFS solution, transferred to 25 ml Erlenmayer flasks, and 8 ml CMFS was added. The flasks were gently shaken for 30 min at 5˚C. The suspensions were thereafter filtered through a 20 µm sieve. Positive controls were prepared by adding 1 M H_2O_2 to flask containing tissue from unexposed clams. The filtered suspensions were centrifuged at 400 g for 5 min. 8 ml supernatant were removed and cells were re-suspended in the remaining 2 ml. The positive controls were prepared by adding 1 M H_2O_2 to flask containing soft tissue from unexposured clams for 10 min exposure. 100 µl ml of the cell suspensions were added to 125 µl LMP agarose, and 100 µl of suspensions were applied to fully frosted slides, pre-coated with a layer of NMP agarose. Cover glasses were applied and slides were cooled on ice for 15 min, in order for the gel to dry. Slides were placed in lysis solution (2.5 M NaCl, 0.1 mM EDTA, 10mM Tris 1% triton X-100) afterwards for at least 1 h in refrigerator. Slides were placed in electrophoresis solution (0.3 M NaOH, 1 mM EDTA), for 10 s in order to remove excess salts. The slides were then placed in electrophoresis chamber for 15 min alkaline unwinding followed by 5 min electrophoresis at 300 mA and 25V. Finally the slides were neutralized two times for 5 min in 0.4 mM Tris. Slides were dried for 15 min and dyed with etidium bromide (20µg/ml) and stored in refrigerator overnight. Numbers on slides were covered in order to ensure random analyses and scored using the software comet Assay III image-analysis from Perspective Instruments. 50 comets were scored for each slide.

2.8 AAS measurements

Sediment and clam samples (i.e., sediment, clams) were prepared and measured on either graphic-AAS or flame-AAS according to methods used by Cong et al. (2011) for Ag or Pang et al. (2012) for Cu. All samples were lyophilized (Christ Alpha 1-2, Osterode, Germany) at -50°C overnight. Afterwards, the welfon tubes with samples and 65% HNO_3 were digested in the micron oven (Milestone MLS-1200 Mega,

Leutenkirch, Germany) at 250 W, 400 W, 600 W and 250 W for 6 min, respectively. The acid suspension was neutralized with 25% NH_4OH for Ag measurements. The detection limits of sediment samples were 0.35 µg Ag/L and 0.15 µg Cu/L.

2.9 Statistics

Data regarding genotoxicity were transformed to the 4^{th} root in order to meet criteria for parametric analysis, as described by Rank et al. (2005), and subsequently analyzed with one way analysis of variance (ANOVA) test. Parametric or non-parametric analysis of variance (ANOVA or Kruskall-Wallis), depending on data homogeneity as tested by Levine's test, was used to test effects of metal in various forms in all experiments. Before choosing the non-parametric test, the possibility of data transformations as a solution for distributional problems was assessed. Linear regression was used to estimate the rate of loss of accumulated Cu over time in Experiment 3. The comparison of simple linear regression was made on k_us and k_ds from different Cu treatments (Zar, 1999). All data in the present study were expressed as the mean value ± 1 standard deviation (1SD). Statistics are presented as p values unless other was stated. Significance level was based on $p \leq 0.05$ and marginally significant was based on $0.05 < p < 0.10$.

3. RESULT

3.1 Metal concentrations in sediments for exposure

In Experiment 1, no significant differences were found in Ag concentrations of different treatments (One-way ANOVA, $p = 0.477$). The measured concentrations were 178.0 ± 13.7 µg/g dw sed for Aqueous Ag ion, 134.4 ± 22.4 µg/g dw sed for AgNPs-20, 174.6 ± 49.1µg/g dw sed for AgNPs-80 and 172.1 ± 48.6 µg/g dw sed for Micron-Ag, respectively. The Ag concentrations were below detection limits in control and in PVP-control sediments.

In Experiment 2 and during the uptake period of Experiment 3, no significant differences were found in Cu concentrations of different treatments (One-way ANOVA, $p = 0.828$). The Cu concentrations were 153.9 ± 9.0 µg/g dw sed in Aqueous Cu ion, 147.7 ± 5.9 µg/g dw sed in CuO NPs and 152.0 ± 18.9 µg/g dw sed in

Micron-CuO, respectively. The Cu concentrations in control and clean sediment for depuration were low but measurable at 4.2 ± 1.0 µg/g dw sed.

3.2 Mortality

In Experiment 1, no mortality was observed after 35 d exposure to Ag amended sediment (data not showed). In Experiment 2, mortality for the different treatments was 4.8 ± 8.2% in control, 2.4 ± 6.3% in Aqueous Cu ion, 2.4 ± 6.3% in CuO NPs and 14.3 ± 15.0% in Micron-CuO, respectively. No significant differences in mortality for these Cu forms were detected (Kruskal-Wallis test, $p = 0.173$). In Experiment 3, the mortality during the exposure period was 4.5% in control, 7.5% in Aqueous Cu ion, 15.5% in CuO NPs and 4.5% in Micron-CuO. And during the depuration period, mortality was 6.7% in control, 10.0% in Aqueous Cu ion, 11.1% in CuO NPs and 2.9% in Micron-CuO. The mortality of clams in CuO NPs treatment was highest in all treatments. However, no statistics can be run on mortality data of Experiment 3 due to the experiment design.

3.3 Burrowing behavior

After first 24 h, burrowing activity in all treatments was showed in **Figure 2**. In Experiment 1, the percentage of unburied clams reached a steady state from 30 min to 60 min with 25.8% (± 19.1%) clams left on sediment surface after 60 min (pooled data for all treatments). There were no significant effects on percentage of unburied clams due to exposed Ag forms after 60 min (One-way ANOVA, $p = 0.429$). In Experiment 2 and during the uptake period of Experiment 3, there were no significant differences in percentage of unburied clams due to exposed Cu forms after 7 h (One-way ANOVA, $p = 0.624$ and $p = 0.388$, respectively). Clams in Experiment 2 burrowed faster than those in Experiment 3 during first 7 h in Cu amended sediment (Kruskal-Wallis, $p < 0.001$). And it should be noted that clams in Experiment 2 were significantly smaller than those in Experiment 3 (Kruskal-Wallis, $p < 0.001$).

3.4 Condition index

In Experiment 1 CI wasn't affected significantly by exposed Ag forms (Fig. 3(a), One-way ANOVA, $p = 0.591$) but decreased significantly overtime (One-way

ANOVA, p = 0.006). In Experiment 2, a significant interaction of Cu form and time was detected (two-way ANOVA, p = 0.006) on CI of exposed clams. The CI of Aqueous Cu ion treatment (1.3 ± 0.4) was greater than CI of CuO NPs (0.6 ± 0.3) or Micron-CuO treatment (0.7 ± 0.1) on day 3 (**Fig. 3**), which may indicate individual differences at the beginning of the exposure since clams were collected from different places. Despite these initial differences, the condition index of all treatments were similar on day 35 with 0.6 ± 0.3 in control, 0.6 ± 0.1 in Aqueous Cu ion, 0.5 ± 0.2 in CuO NPs and 0.6 ± 0.2 in Miron-CuO, respectively (n = 7). Hence, a decrease in CIs was due to exposure time (One-way ANOVA, $p < 0.001$) but not related to Cu forms (ANCOVA, p = 0.674).

3.5 Weight-specific body burden of metals in soft tissue after exposure period

All forms of Ag were bioavailable to clams (**Fig. 4**). In Experiment 1 significant differences of Ag weight-specific body burden (WSBB) in soft tissue for different Ag forms were detected on day 35 (One-way ANOVA, p = 0.005). The specific body burden of Ag was significantly lower in the soft tissue of clams in the micron-Ag treatment relative to the Aqueous Ag ion and AgNPs-20 treatment (Tukey's test, p = 0.004 and p=0.027). For control and PVP-control clams accumulated Ag concentrations in soft tissue were below detection limit of graphite-AAS (0.18 µg Ag/g dw soft tissue).

In contrast, in Experiment 2 there were no significant differences due to Cu forms in the WSBB of Cu in soft tissue of clams (Fig. 4b, One-way ANOVA) even though there appeared to be a trend that WSBB of Cu in soft tissue decreased with increasing metal particle size (**Fig. 4**). The WSBB in soft tissue was 111.5 ± 50.7 µg/g dw soft tissue in control group (data pooled over time, n = 3 per time, and 18 clams in total). It increased approximately 12 times, 10 times and 5 times in treatments of Cu ions, CuO NPs and Micron-CuO, respectively.

In the uptake period of Experiment 3 there were, however, significant effects of exposed Cu forms on WSBB of Cu in soft tissue of clams (One-way ANOVA, p = 0.011). Although, the WSBB of Cu from Micron-CuO (95.2 ± 31.2 µg Cu/g dw tissue)

was twice to control (41.9 ± 20.2 µg Cu/g dw tissue), the difference was not significantly different from each other (Tukey test, p = 0.993). This indicates that Micron-CuO was not bioaccumulated to any degree in Experiment 3.

3.7 Net uptake (Experiment 2)

In soft tissue, the WSBB increased over 35 days' exposure (**Figure S1**), which was reflected by the positive k_us in different Cu treatment (**Table 1**). The k_u of Cu was significantly higher in Aqueous Cu ion treatment followed with CuO NPs treatment, and it was the lowest in Micron-CuO (F[3, 45]=3.39). Given that both WSBB and k_us increased, no trends towards steady-state body burden levels were observed for the exposure to Cu in different form in the present study.

In shell, the measured Cu in shells reached a peak at different times (**Fig. 5**). From day 7 to day 15, it increased approximately twice for control, 4 times for Aqueous Cu ion, 3 times for CuO NPs and 3 times for Micron-CO compare to the background concentration of Cu in shell on day 0. However, the k_us of Cu was much lower than uptake in soft tissue.

3.8 Depuration (Experiment 3)

In soft tissue, the depuration of Cu in soft tissue was only observed in the Aqueous Cu ion treatment with a negative k_d (**Fig. S2, Table 2**) and WSBB decreased 65.5% after 15 days in clean sediment. The WSBB of Cu did not decrease significantly in CuO NPs or Micron-CuO (One-way ANOVA, p = 0.305 and p = 0.437) and most Cu was remaining in soft tissues after 15 days. A significant high k_d was detected in Aqueous Cu ion than CuO NPs and Micron-CuO (F [3, 45] = 3.39).

In shell, *n*o measurable reduction of WSBB of Cu in shell was detected (One-way ANOVA, p >0.05), of which most Cu was left after 15 days except for the Micron-CuO treatment. The WSBB of Cu in Micron-CuO decreased significantly (One-way ANOVA, p = 0.002), which 43% Cu remaining after 15 days. In addition, no significant differences were detected in k_ds of shell (F [3, 43] = 5.47).

3.9 Comet Assay

No significant genotoxic effects were observed for any of the Cu exposed specimens

(n = 4) (one way ANOVA, p = 0.160) upon experimental termination. The average tail moment was approximately 10% in all Cu treatments (Fig. 5). However, exposing *M. balthica* cells to H_2O_2 (positive control) did lead to significant DNA damage (one way ANOVA, p =0.02 when the positive control was included) indicating that the lack of effect was not due to an experimental artifact.

4. DISCUSSION

4.1 Effects of metals

Both Ag and Cu are two of the most toxic metals to invertebrates that have been documented in marine and estuarine environments, but the toxicity ranking of these two metals is species specific (Bryan, 1984). For *M. balthica*, the 50% mortality concentration of Cu ions (added as $CuSO_4$) was in a range of 210 to 1290 mg/L in a seawater (25‰, pH 7.9) exposure for 11 days (Luoma et al., 1983). Unfortunately, no toxicity parameters of Ag ions have to our knowledge been reported for this species. Toxicity studies with Ag ions and Cu ions in sediment is complicated by many factors, such as organic matter, active volatile sulfur and species tolerance (Wang, 1987), which means that it is difficult to compare toxicity parameters among studies. In the present study, the exposure to sediment associated Aqueous Cu ion caused less than 10% mortality of *M. balthica*. It suggests that Aqueous Cu ion may be more toxic for this species than Aqueous Ag ion during 35 days exposure. The low mortality found in both aqueous ions may due to the high tolerance ability of *M. balthica* through effective detoxification pathways (see section 4.2).

In the present study, no effects of Ag or Cu form were detected on conditional index CI. Stresses caused by metal exposure can consume energy, affect net glycogen accumulation, and reduce CI of clams. CI also related to population origins. Different sensitivities of CI to metal exposures were found in clam populations from different locates (Hummel et al., 1996,Sokolowski et al., 1999,Hummel et al., 1997,Luoma et al., 1983), which may due to differences of genotype of tested clams. CI of clams from Somme decreased during water exposure to Cu up to concentration 36 µg/L; while it increased in clams from Gironde at the same concentration (Hummel et al.,

1996). The difference of genotype of *M. balthica* populations cannot be excluded from its interference to CI in the present study. In addition, food amount may also affect the effect of metal exposure on CI of bivalve (Martel et al., 2003,Bervoets et al., 2005). The difference in food amount did not seem to be a factor to the effect of metal exposure on CI in the current study because of the similar sediment amount in each replicate from the same experiment.

In the present study, no impacts of Ag or Cu in different forms were detected on the burrowing activity at the beginning of the exposure. Impairment of burrowing behavior has previously been observed in *S. plana* after exposure to both Cu ions and CuO NPs at the concentration 10 µg/L for 16 days (Buffet et al., 2011). In the present study, a short duration of up to 24 hours was not long enough to observe any effects of Ag and Cu exposure on the burrowing behavior. De Wilde (1975) found a faster burrowing behavior of small clams than big ones due to a fast metabolic rate and high oxygen consumption. Clam size dependent burrowing activity was observed in the present study as well during the first few hours in Cu amended sediment.

There were no observed genotoxic effects to *M. balthica* after Cu exposure in Experiment 3. This was somewhat surprising since other studies have demonstrated genotoxic effects of Cu at lower exposure concentrations for a comparable organism, *S. plana* (Petridis et al., 2009). The lack of effect was not due to sensitivity of the assay, since positive controls showed significant effects when compared to negative controls. Furthermore, the negative controls had values that were in accordance with that found in comparable studies (Al-Subiai et al., 2011). One possible reason for the lack of effect could be the activation of repair enzymes in organisms during the exposure period. Bihari et al. (1990) showed that marine bivalves do have such repair organisms. This could explain the difference between the Cu exposed clams and the positive controls, since the latter were made by exposing cells *in vitro*. Tumor suppressing genes involved in DNA repair have been identified in the marine clam *Mya arenaria* (Holbrook et al., 2009) illustrating that this group of animals does have the capacity to cope with and repair DNA damage. Further studies are needed in order

to verify whether repair mechanisms do pose an important role in regard to *M. balthica*'s ability to cope with genotoxic effects of Cu. This is relevant for the future usefulness of DNA damage as an ecotoxicological endpoint that has otherwise been shown to be sensitive to effects of metal NPs (Cong et al., 2011).

4.2 Bioaccumulation

In the present study, increased body burden of Ag and Cu suggested metals in all forms were bioaccumulated in *M. balthcia* after 35 days of exposure. Ratios of body burdens to sediment concentrations of metals were approximately 2 for aqueous Ag ions (clam size of 11.0 ± 2.0 mm), approximately 12 for aqueous Cu ions in clams of 9.6 (± 1.4) mm and 6 for aqueous Cu ions in clams of 12.0 (± 1.0) mm. In the field, the bioaccumulation factor of *M. balthica* can vary from 0.04 to 60 for Ag and from 0.05 to 8 for Cu based on metal concentrations in soft tissue (Luoma et al., 1983, Luoma et al., 1991). We did not measure whether body burdens reached steady state in Paper IV and it is likely they did not. So the body burden of Ag and Cu may have increase to even higher levels than I observed. However, the body burden of both aqueous ions (i.e., Cu and Ag) was extreme high compared to body burdens of clams from unpolluted sites (Cu: up to 300 µg/g dw tissue; Ag: up to 100 µg/g dw tissue from Luoma et al. (1991)) which may be related to a very high exposure concentration in the experiment (Cu: up to 200 µg/g dw sed; Ag: up to 200 µg/g dw sed) compared to unpolluted sites (Cu: up to 100 µg/g dw sed; Ag: up to 2.5 µg/g dw sed in Luoma et al. (1991)). Such high body burdens of Ag and Cu without detected toxicity, especially in small clams (Experiment 2), may be due to the strong adaptive ability of the species as a consequence of metal detoxification. Adult bivalves seem to be able to detoxify the accumulated Ag and Cu ions by binding them to metallothionein/-like proteins and by enclosing them in sulfide-rich granules and basal membranes of cells (George et al., 1986, Johansson et al., 1986). In addition, extreme high Cu body burden in small clams can be detoxified by inducing metallothionein/-like proteins at higher concentration as evidence from observed negative relationship between organism size and metallothionein/-like proteins

concentration (Bordin et al., 1997, Mouneyrac et al., 2000).

Body burdens of Cu from Cu NPs and micron CuO particles were also higher in small clams than in big clams. Strong and Luoma (Strong and Luoma, 1981) found size dependent uptake rate in some populations of *M. balthica* from San Francisco Bay. It was also confirmed by Lee et al. (1998), that weight-specific influx rate of cadmium were negatively related to the size of *M. balthica*. This may be the case for big clams with a lower uptake rate than small clams in Paper IV. However, effects of organism size on bioaccumulation of CuO in different forms need to be further investigated in other experiments.

A form dependent net uptake of Cu and Ag was observed in the present study, such that there was a trend that k_u decreased with increasing particle size. This may be related to the particle sorting commonly existing in bivalves. During exposure to glass wool (silicon dioxides, 3-7 μm length and 0.18-1 μm), particle sorting has been observed in the bivalve, *Mytilus edulis* where larger fibrils were found in gill epithelial cells at high amounts, and only small and fine particles (up to 200 nm) entered the primary tubules after 12 h and appeared finally in the secondary tubules after 24 h (Koehler et al., 2008). In addition, a longer gut retention time of polystyrene nanoparticles than 10 μm beads indicated that most nanoparticles were taken up in the digestive gland via endocytosis (Ward and Kach, 2009). No dissolution of CuO NPs has been detected by Buffet et al. (2011) in marine sediment (15‰) amended with CuO NPs from the same producer as used in the present study. Otherwise, the k_us of CuO particles reported here would be overestimated if dissolution occurred in the sediment.

Uptake of Cu ions involves transportation across the cell membrane through available protein carriers (Luoma, 1983); while uptake of NPs occurs via endocytosis (Conner and Schmid, 2003). Endocytosis rates of single-walled carbon nanotubes (50 nm) and gold nanoparticles (50 nm) have been determined to be 10^{-3} min^{-1} (equal to 1.440 d^{-1}) and 10^{-6} min^{-1} (equal to 0.001 d^{-1}), respectively (Wang et al., 2010), which is comparable to the rates measured in the present study (i.e., 0.464 d^{-1} for CuO NPs;

0.091 d^{-1} for micron CuO). In addition, different endocytotic pathways and uptake rates have been displayed in direct internalization of nanoparticles in different cells. Positive charged nanoparticles are preferentially internalized into in HeLa (cervical cancer cell line) cells by the clathrin-mediated endocytotic pathway at a higher uptake rate than negative charged nanoparticles (Harush-Frenkel et al., 2007). Uptake rate of negative charged cerium oxide nanoparticles was found higher than positive charged nanoparticles in adenocarcinoma lung cells (Patil et al., 2007). In a study of cellular internalization pathways of nanoparticles by Gratton et al. (2008) found that lager particles of 50-100 nm were internalized through the caveolae-mediated endocytotic pathway. Thus, different uptake pathways and cell lines may be involved in the internalization of CuO NPs and micron CuO particles in the present study.

In the present study, the k_d for Aqueous Cu ions (7.4% d^{-1}) was close to a typical depuration rate constant of 5% d^{-1}, which was described best by a one-compartment model (cited in (Griscom et al., 2002)). As mentioned above, accumulated Cu ions are sequestered in cytosolic proteins (i.e., metallothionein/-like proteins) followed by transportation to lysosomes for degradation into insoluble residual bodies, which are excreted out of organisms (Amiard et al., 2006, Wang and Rainbow, 2010). There was no significant decrease of accumulated Cu from CuO NPs in Paper IV.

Difference in loss dynamics suggested that the form of Cu accumulated in *M. balthica* from sediment associated CuO NPs was probably not Cu ions. The depuration mechanisms of accumulated CuO NPs were not very clear. However, a different detoxification pathway of CuO NPs was implied by a significant association of Ag NPs with organelles and metal-rich granules in polychaete, *N. diversicolor* (García-Alonso et al., 2011). Compartmentalization of accumulated CuO NPs should be studied further in *M. balthica* to understand its strategies for ENPs detoxification.

Incorporation of metals into bivalve shells commonly involves two sources: 1) metals accumulated in the soft tissue of bivalves may be transferred to the shell during shell deposition from mantle tissue (Jodrey, 1953), and 2) adsorption onto shell surface from the surrounding external environment. Cu accumulated in shells of *S. plana* was

shown to be a result of passive absorption of Cu in seawater (Bryan and Uysal, 1978). Since no growth of shells was occurred during exposure in the present study, the bioaccumulation of Cu in the shells (including control clams) is thus most likely due to the absorption of different Cu forms from the sediments onto shell surfaces. As such, this form of bioaccumulation likely has little impact on the clam itself. However, it may still be of importance for bioavailability to predators of clams and thus to trophic transfer of metals including ENPs.

5. CONCLUSION

No adverse effects on *M. balthica* exposed to sediment-associated Ag and Cu in various forms, including NPs, were observed. The exposure level used in the present study, especially for aqueous ions, was within the range of Ag and Cu in the environment based on the worst scenarios of 100 µg Cu/L and 260 µg Ag/L in aquatic environmental (US EPA, water criteria), which was corresponding to 1 ~10 g Cu/ kg dw sed and 3~30 mg Ag/g dw sed based on equilibrium partitioning model with typical metal partitioning coefficients of 10^4~10^5 (Balls, 1989). Differences in bioaccumulation and the corresponding bio-kinetic parameters depending on metallic form were detected for both Ag and Cu; however, depuration of CuO NPs was very slow compared to aqueous Cu ions, but the specific reasons for this observation could not be determined in this study. Differences in detoxification and internal compartmentalization of accumulated Cu, both possible explanations for this difference, were not explicitly investigated. More studies are needed to understand the cellular compartmentalization of ENPs.

Acknowledgement

This work was supported by Roskilde University (RUC) and EU NanoReTox project (7^{th} Frame work Programme, Grant Aggreement no. CP-FP 214478-2). We give our sincerely thanks to National Histroy Museum (NHM, London) for particle supplyment, and we also thank our lab technicians, Anne-Grete Winding, Michael Rabe and Anne Faarborg in ENSPAC-RUC for their help during experiment.

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FIGURE LEGENDS

Fig. 1. Collecting locations of *Macoma balthica* in Denmark for the different experiments.

Fig. 2. Percent unburied clams present on the sediment surface versus time in (a) Experiment 1 (n=20); (b) Experiment 2 (n=42) and (c) Experiment 3 (n=42). Error bars stand for 1 standard deviation (only shown for time = 420 min and 1440 min).

Fig. 3. Condition index of exposed clams (a) in Experiment 1 at day 0 and day 35 (n=20) and (b) based on tissue dry weight in Experiment 2 during exposure period (n=42). Error bars stand for 1 standard deviation.

Fig. 4. Weight-specific body burden of exposed metals in clams after the exposure period for the different experiments (n=3). Error bars stand for 1 standard deviation. Stars refer to a significant difference from other treatments (explained in the text).

Fig. 5. DNA damage expressed as % DNA in the tail from the Comet assay for Experiment 3 (n=4). Error bars stand for 1 standard deviation.

TABLE

Table 1. Net uptake rates (k_u, d^{-1}) in *Macoma balthica* for different time intervals during exposure to Cu in various forms in Experiment 2 (n=3).

Clams	Day	Control	Aqueous Cu ions	CuO NPs	Micron-CuO
Soft tissue	3	-0.308	0.415	0.103	-0.084
	7	0.154	0.466	0.102	0.088
	11	-0.008	0.403	0.215	0.002
	15	0.062	0.400	0.341	0.040
	23	0.044	0.669	1.552	0.139
	35	-0.015	1.485	0.470	0.362
Average k_u		-0.012	0.640	0.464	0.091
Shell	3	-0.015	0.000	0.001	0.011
	7	0.003	0.016	0.087	0.087
	11	0.077	0.065	-0.052	0.010
	15	-0.014	0.011	-0.002	-0.034
	23	-0.027	-0.033	0.000	0.001
	35	-0.003	0.008	0.000	0.015
Average k_u		0.004	0,011	0.006	0.015

Table 2. Depuration rates (k_d, d^{-1}) of accumulated Cu in various forms in *M. balthica* during the depuration period in Experiment 3 (n=1 or 2) estimated from the slope of linear regression (Fig. 6).

Forms	Soft tissue				Shell			
	kd	p	r^2	t½	*kd*	p	r^2	t½
Control	-0.032	0.171	0.114	-[a]	0.009	0.514	0.027	-
Aqueous ion	-0.074	0.006	0.423	9	-0.020	0.178	0.126	-
CuO NPs	-0.030	0.305	0.066	-	0.014	0.343	0.060	-
Micron-CuO	0.019	0.437	0.044	-	-0.053	0.002	0.509	13

a. '-' refers that t½ cannot be determinded because of insignificant linear regression of k_d.

FIGURE

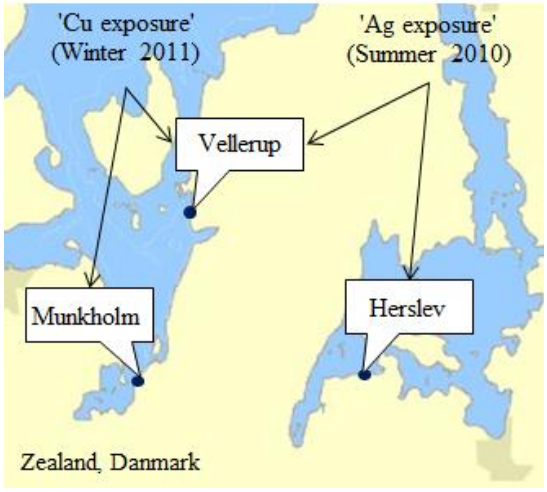

Fig. 1

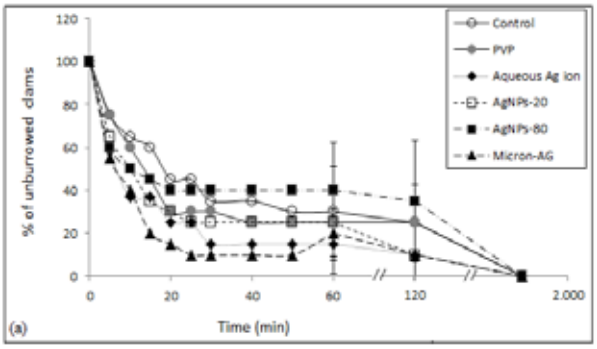

Fig. 2(a)

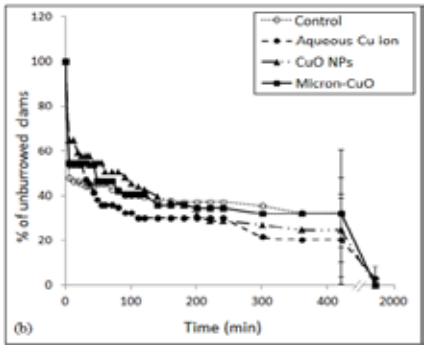

Fig. 2(b)

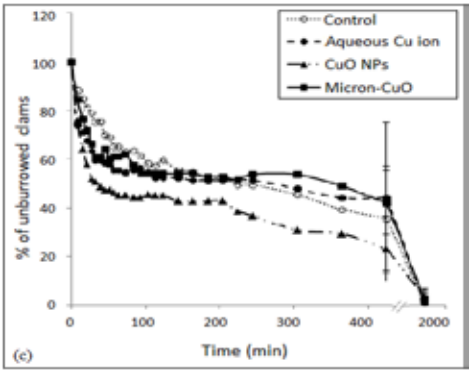

Fig. 2(c)

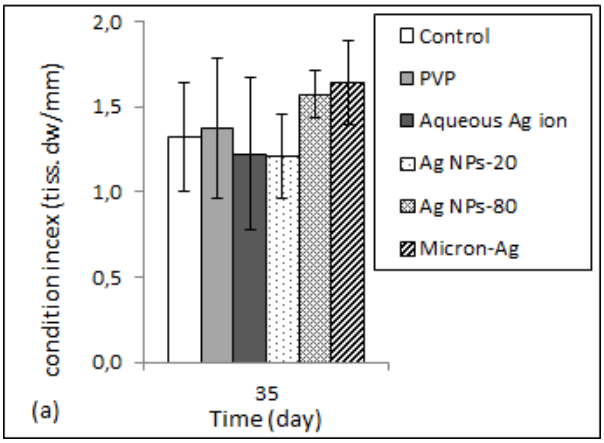

Fig.3(a)

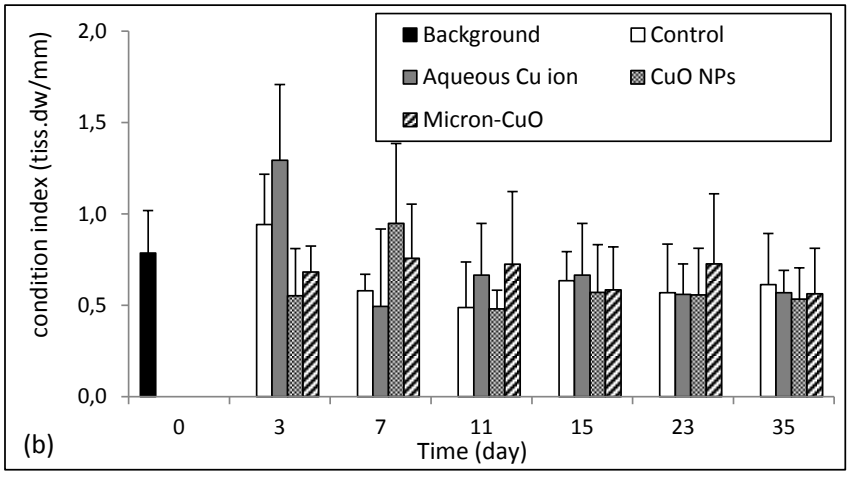

Fig.3(b)

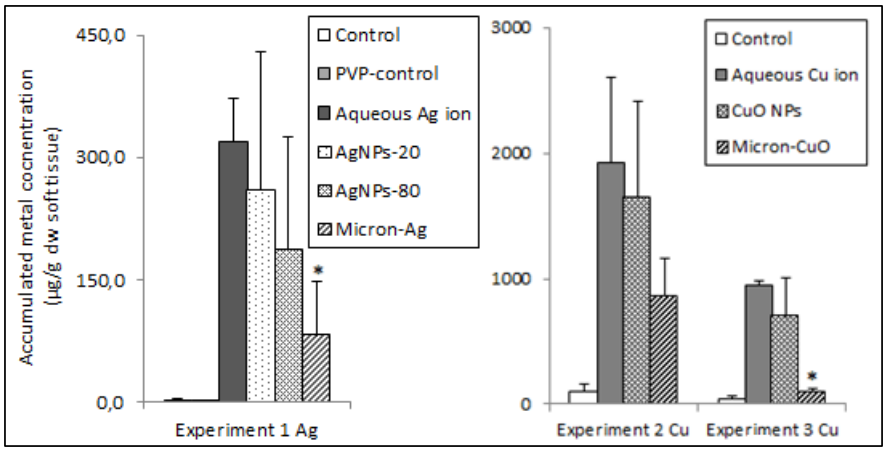
Accumulated metal cocnentration (µg/g dw soft tissue)
450,0
300,0
150,0
0,0
Control
PVP-control
Aqueous Ag ion
AgNPs-20
AgNPs-80
Micron-Ag
*
Experiment 1 Ag
3000
2000
1000
0
Control
Aqueous Cu ion
CuO NPs
Micron-CuO
*
Experiment 2 Cu
Experiment 3 Cu

Fig.4

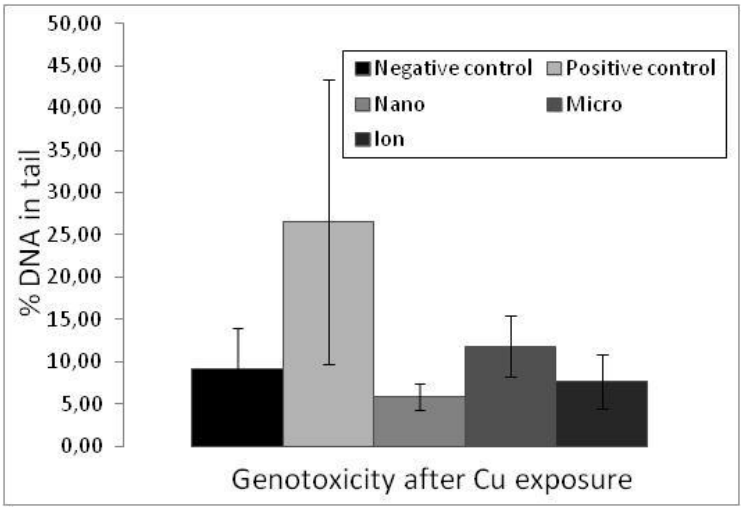
% DNA in tail
50,00
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
Negative control
Positive control
Nano
Micro
Ion
Genotoxicity after Cu exposure

Fig. 5

SUPPLYMENTAL INFORMATION

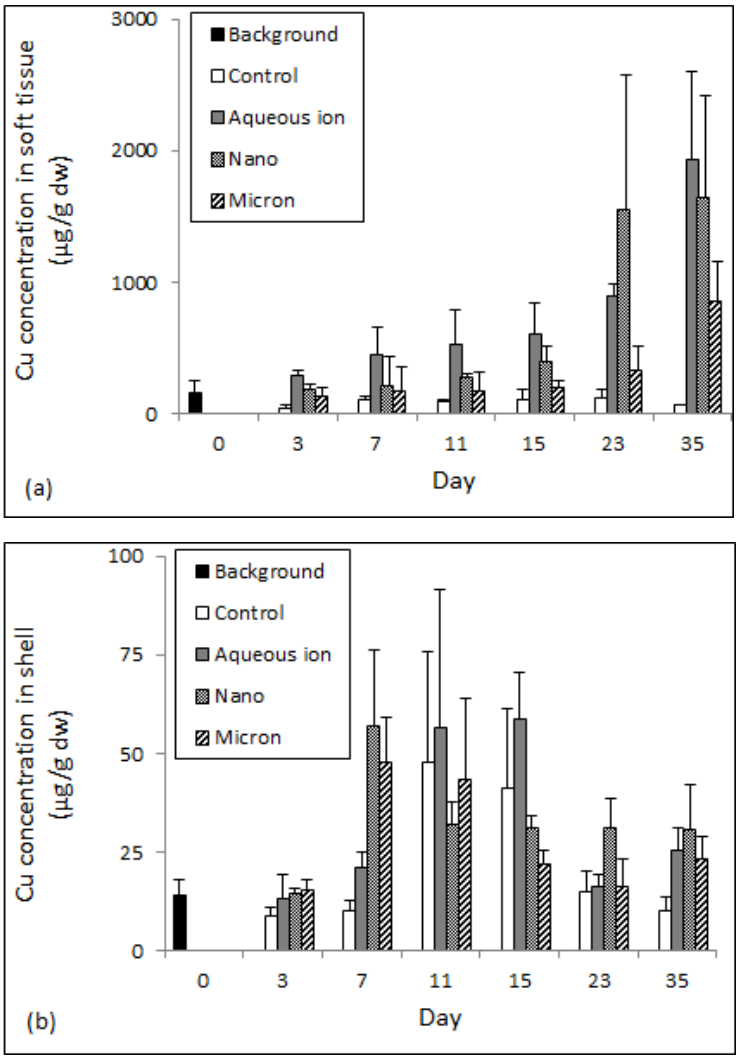

Figure S1. Weight-specific body burden of Cu in (a) soft tissue and (b) shell of clams over time in Experiment 2 (n=3). Error bars stand for 1 standard deviation.

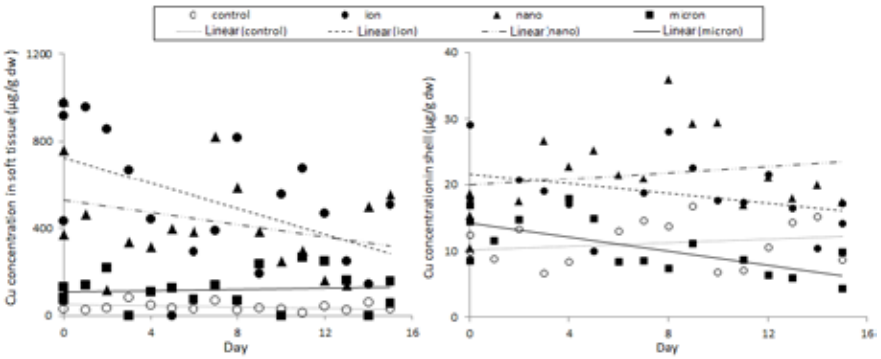

Figure S2. Linear regression of weight-specific body burden of Cu in various forms in (a) soft tissue and (b) shell overtime during depuration period in Experiment 3 (n=3). Error bars stand for 1 standard deviation.